

**HSV-1 ICPO-MEDIATED IMPAIRMENT OF HOST INTRINSIC AND
INNATE IMMUNE FACTORS**

By

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**HSV-1 ICPO-MEDIATED IMPAIRMENT OF HOST INTRINSIC AND INNATE
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ABSTRACT

Understanding mechanisms viruses use to evade the immune system and to persist within its host, is of crucial relevance in controlling, preventing and treating HSV-1-associated diseases. As reviewed in this dissertation, ICP0 is a key viral regulatory protein that modulates the switch between lytic and latent cycles of the virus. ICP0's role in stimulating viral replication is mediated, in part, by its impairment of the host defenses.

The purpose of the current study was to undertake structure/function analyses of ICP0 that will allow us to begin to delineate how ICP0 promotes the dispersion and/or degradation of the antiviral ND10 components and how an established IFN response is disarmed by HSV. From our PML studies, we have defined two overlapping regions within the central N-terminal portion of ICP0 (residues 1 to 311) that promote the dissociation and degradation of PML and dissociation of Sp100 (residues 1 to 427). Additionally we found that the first 388 N-terminal amino acids of ICP0 play a role in impairing the IFN response, with the residues spanning from 212-388 being important for this activity. Overall these N-terminal regions of ICP0 play a vital role in impair

two host defenses, ND10 and IFN β . Results from these studies may be used to design therapeutic interventions that limit HSV-1 infections and diseases.

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List of Abbreviations

HSV	Herpes simplex virus
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
ICP0	Infected cell protein 0
ORF	Open reading frames
PML	Promyelocytic leukemia protein
Sp100	Speckled protein of 100 kDa
hDaxx	Human death domain-associated protein 6
ND10	Nuclear domain 10
ATRX	Alpha thalassemia/mental retardation syndrome X-linked
MFI	Mean Fluorescent intensity
Wt	Wild type
IFN	Interferon
Ub	Ubiquitin

IE	Immediate early protein
E	Early protein
L	Late protein
LAT	Latency associated transcripts
Oct1	Octamer transcription factor 1
HCF	Host cell factor
RING	Really interesting new gene
SUMO	Small ubiquitin-like modifier
SIM	SUMO interacting motif
SLS	SUMO like sequence
NLS	Nuclear localization signal
STUbL	SUMO targeted Ubiquitin ligase
ISGs	Interferon stimulated genes
	Carbobenzoxy-Leu-Leu-leucinal
MG132	(proteasome inhibitor)
HEL	Human Embryonic Lung cells

HSK	Herpes stromal keratitis
hpi	Hours post infection
dpi	Days post infection
MOI	Multiplicity of infection
ACV	Acyclovir
CNS	Central nervous system
TK	Thymidine kinase
IFI16	IFN γ -inducible protein 16
MYD88	Myeloid differentiation factor 88
Mal	MyD88 adaptor-like protein
BSA	Bovine serum albumin
PBS	Phosphate-buffered saline
GFP	Green fluorescent protein
FBS	Fetal bovine serum
NK	Natural killer
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell

TLR	Toll Like Receptor
RIG-I	Retinoic acid Inducible gene I
MDA5	Melanoma differentiation Antigen 5
ISRE	Interferon stimulated response element
GAS	IFN- γ -activated site
JAK	Janus activated kinase
STAT	Signal transducer and activator of transcription
TYK2	Tyrosine kinase 2
ISGF3	IFN-stimulated gene (ISG) factor 3
BHV-1	Bovine herpes virus type 1
EHV-1	Equine herpes virus type 1
PRV	Pseudorabies virus
VZV	Varicella-zoster virus

1. CHAPTER I: INTRODUCTION

Adapted in part from: “HSV-1 ICP0: An E3 Ubiquitin Ligase That Counteracts Host Intrinsic and Innate Immunity”

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1.1. HERPES SIMPLEX VIRUS TYPE 1

1.1.1. Significance, Epidemiology, Diseases and Treatments

HSV-1 (herpes simplex virus 1) is a ubiquitous human pathogen that infects about 80 % of the world population. Hallmarks of the infection by HSV-1 are its ability to establish a lifelong latent or quiescent infection in the sensory neurons, following an initial productive or lytic infection in mucosal or epidermal cells [3] (Figure 1-1) and its ability to repeatedly reactivate over time [4,5]. A wide range of stimuli, one example being stress, can trigger the switch from latent to lytic infection in neurons of the trigeminal ganglia [6]. Upon reactivation the virus initiates a productive cycle at the primary site of infection, with the potential to spread to nearby epithelial and mucosal cells [7].

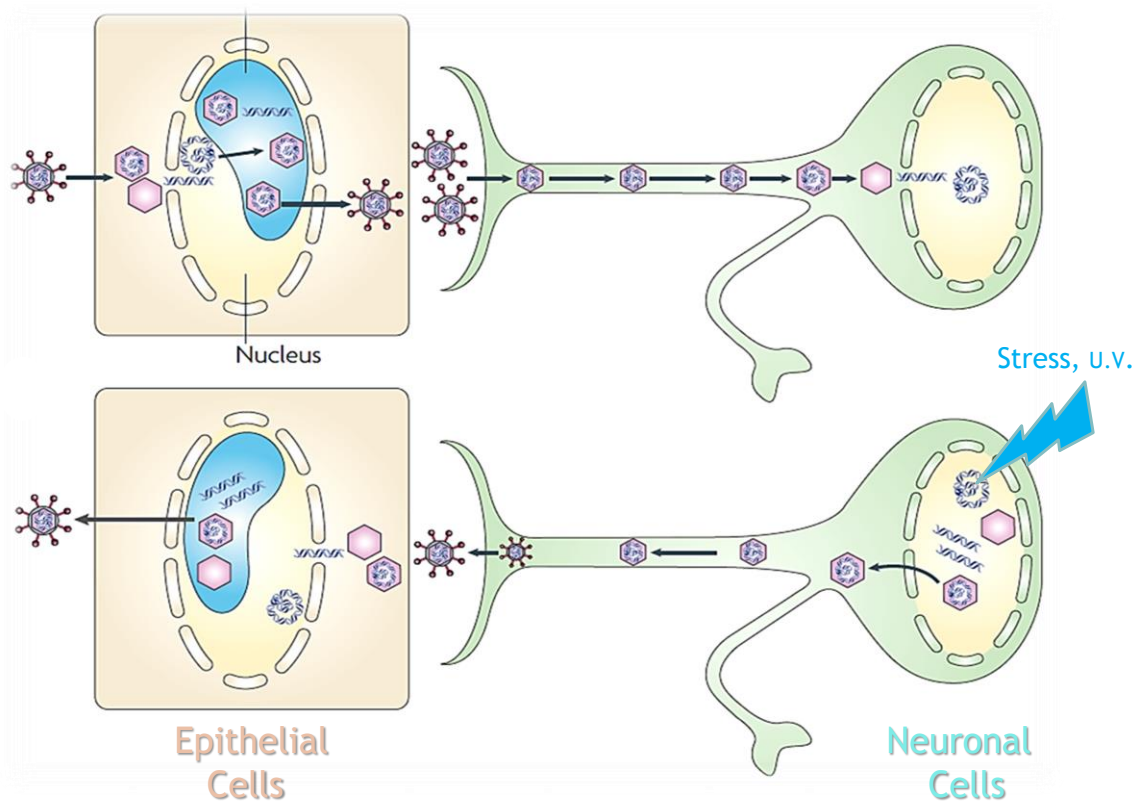


Figure 1-1. Herpes simplex virus latency and reactivation. From the initial site of infection in the mucosal surface the virus enters the innervating neurons from the trigeminal ganglion and via retrograde microtubule-associated transport accesses the neuronal cell body. DNA persists in the neuronal nucleus in a circularized form and expresses the latency associated transcripts (LATs). Latency is characterized by the presence of DNA and absence of infectious virus. Different stimuli can induce the reactivation of viral lytic infection leading the virion transport back to the primary site of entry resulting in a recurrent infection. ICP0 plays a central role determining whether an infection will be productive or latent. (Adapted from Nat Rev Microbiol. 2008 Mar;6(3):211-21 [1]).

Herpetic infections range from fever blisters or vesicular eruptions around the mouth, called cold sores, to blindness and encephalitis [6]. More than 95 % of the ocular infections in adults are due to HSV-1 [8]. Infection of the cornea can lead to herpes stromal keratitis (HSK) a potential blinding disease that results from recurrent inflammation and corneal scarring [9,10].

Although rare, encephalitis caused by herpes, accounts for 40 to 50 % of the total cases of encephalitis [6] and has a mortality rate of almost 70% in untreated individuals and leaves severe sequelae in survivors; 90% of herpetic encephalitis cases is due to HSV-1, with the remaining 10% caused by HSV-2 [11].

HSV-1 can also infect the genitals. Genital lesions are often very painful and have been associated with high rates of morbidity and mortality. HSV-1 has been historically associated with oro-facial infections while HSV-2 has been more frequently associated with genital herpes. Nevertheless increases in the prevalence of HSV-1 as causative agent of herpetic genital infections has been reported in the past years versus HSV-2 [12,13], accompanied by an overall decline in the HSV-1 seroprevalence in young populations [14] this decline was suggested to be related to increase in genital infection by HSV-1 resulting from the lack of exposure early in life.

HSV poses a severe risk to immunocompromised individuals [15] because of its high mortality and morbidity [6] as opposed to self-limiting diseases observed in immunocompetent individuals. In immunocompromised individuals HSV can spread to the brain and central nervous system (CNS) causing lethal encephalitis [16].

Although rare, vertical neonatal HSV infections occurs from mother to child during birth and are often asymptomatic, if left untreated in some neonates it can be fatal [17] and those infants that survive severe infections are left with significant brain damage [17-19].

Acyclovir (ACV) is the first line of treatment for HSV-1 infections [20,21] and acts as a competitive inhibitor of DNA polymerase. ACV is a guanosine analogue that undergoes three phosphorylation steps to become active. Initially it is phosphorylated by the viral thymidine kinase (TK) then phosphorylated by cellular kinases, and finally recognized by the viral DNA polymerase [22], Notably ACV can only limit lytic infection but does not eliminate the virus from an infected cell.

Lengthy antiviral treatment are required to manage immunocompromised individuals with a potential issue that such prolonged treatments may promote the emergence of new drug-resistant viral strains [20,21,23,24].

Other than ACV, there are no other effective treatments, such as a commercial vaccine against HSV-1 to prevent infection or reactivation of latent virus.

1.1.2. Viral Structure and Lytic Replication

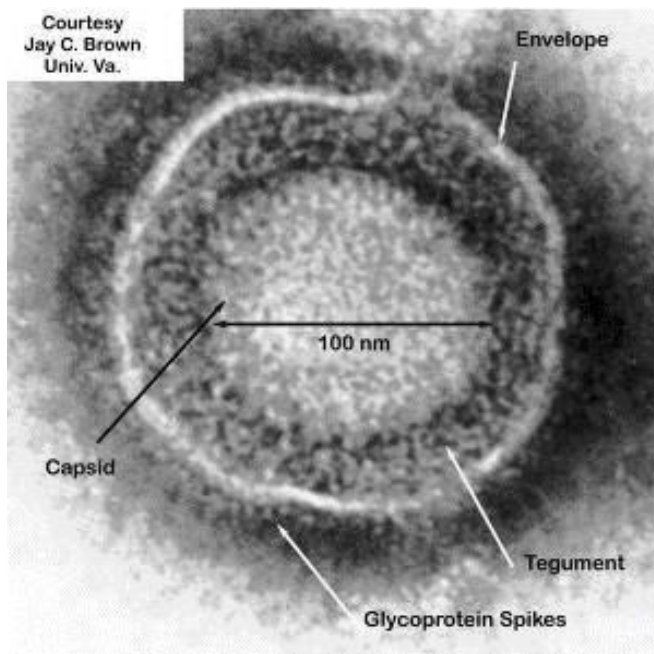


Figure 1-2. HSV structure

HSV-1 virion contains a lipid bilayer called envelope, which is embedded with viral glycoproteins and serves in viral attachment, fusion, entry, and spread. Inside the outer envelope is the icosahedral nucleocapsid, surrounded by an amorphous tegument

comprising of cellular and viral proteins (Figure 1-2). The viral proteins

incorporated in tegument are mainly involved in early events of the viral life cycle and counteract host defenses. The linear ds DNA viral genome is contained within the capsid, and is 152 kb in length [6].

The viral genome contains over 80 open reading frames or ORFs. Although the majority of these genes do not have introns, a small subset of viral transcripts are spliced. There is also a group of non-coding RNA that are expressed during latency, known as the latency-associated transcripts or LATs [5,25]. Overall the genome is composed of two linear fragments of DNA that are covalently linked to one another enclosed by short and long inverted repeats [6,26].

A hallmark of the herpes-virus family is their temporal cascade of gene expression. Three categories of transcripts and proteins are sequentially express: immediate early proteins (IE also designated as α), early (E or β) and late (L or γ) proteins (see Figure 1-3). Their promoter regions differ in their degree of complexity. The IE promoter regions are fairly complex sequences and contain specific DNA consensus transcription factor binding motifs upstream of the TATA box. IE genes are activated by binding of three factors: cellular Oct1 and HCF and the viral tegument protein VP16 [27]. Five IE genes encode the viral proteins: ICP0 (infected cell protein 0), ICP4, ICP22, ICP27 and ICP47 [27]. These IE proteins are involved in converging functions

that promote the establishment of a lytic infection by counteracting cellular antiviral defenses such as viral genome silencing mechanisms and stimulating the expression of the E genes [28]. E genes are primarily involved in viral genome replication, which stimulates the expression of the L genes. The L proteins function in virion related to assembly and egress or are structural components of the virions. The envelope contains many embedded viral glycoproteins and as the viral egresses from the cell it concurrently acquires its tegument [6].

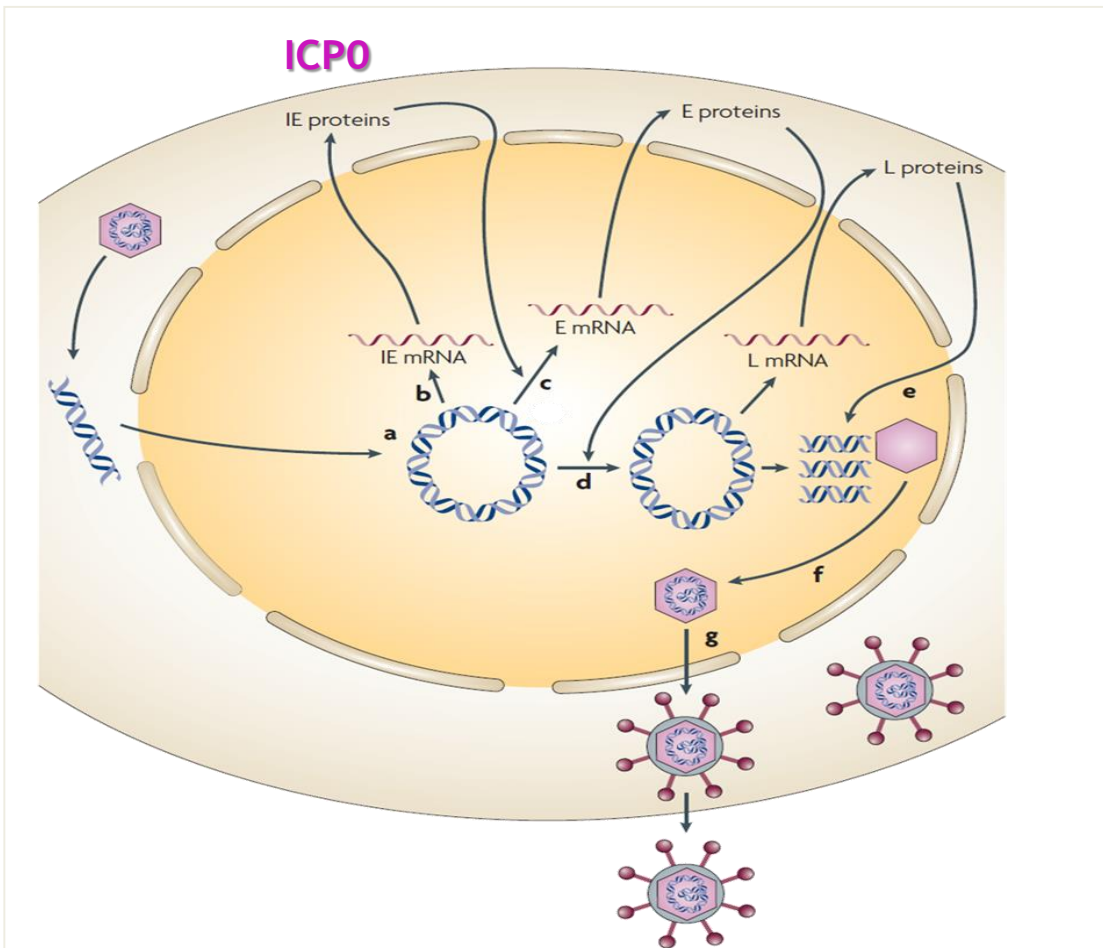


Figure 1-3. HSV-1 lytic life cycle. The HSV-1 lytic life cycle starts with the fusion of the viral membrane to the cell's membrane and transport of the viral DNA to the nucleus which is rapidly circularizing followed by a characteristic temporal cascade of viral gene expression. Immediate early (IE) genes are expressed first, IE proteins stimulate expression of early (E) genes. E proteins together with viral DNA replication promote expression of the late (L) genes. Finally the viral capsid assembles and is encapsidated into virions that egress the cell to complete the infectious cycle. (Adapted from [1] Nat Rev Microbiol. 2008 Mar;6(3):211-21)

1.2. ICP0

HSV-1 encodes the IE protein ICP0 which functions as an E3 ubiquitin ligase. In addition to its E3 ubiquitin ligase activity, ICP0 contains several functional domains and activities (see Figure 1-4). ICP0 is a promiscuous viral transactivator of all three classes of HSV-1 genes. It is important for efficient lytic viral replication, especially in low multiplicity of infection conditions and has an essential role in regulating the switch between the lytic and latent states of HSV-1 [29]. Mutants lacking ICP0 reactivate with reduced efficiency in murine models of viral latency and reactivation [30] and expression of ICP0



Figure 1-4. Functional domains of ICP0. Schematic of WT ICP0, expressed from WT HSV-1. Selected domains and their amino acid residue boundaries are shown: a RING-finger motif, a central transactivation domain, a nuclear localization signal (NLS), a C-terminal transactivation domain (TRANS), which includes an ND10 localization sequence (ND10 – loc). Other domains include phosphorylated region I (224-232), SLS-4 (asterisk, 362-364), phosphorylated region II (365-371), a SIAH-1 binding site (400-410), and a USP7 binding site (618-638).

by adenoviral vectors has been shown to induce reactivation in primary cell culture of latently infected cells [31]. These observations support the importance of ICP0 in reactivation.

In addition, ICP0 is known to impair the activation of the type I interferon (IFN) response through regulatory factors by affecting the levels or activity of many of these proteins amongst which we find IFI16 (IFN γ -inducible protein 16), MYD88 (myeloid differentiation factor 88) and Mal (MyD88 adaptor-like protein).

1.2.1. ICPO's E3 Ubiquitin Ligase Activity

Ubiquitination is an important regulator of protein function and stability within cells [32,33]. This system is regulated by an E1-E2-E3 enzymatic cascade that catalyzes the conjugation of ubiquitin (Ub) to target proteins while conferring substrate specificity. Ub monomers are activated by the E1 enzyme, covalently linked to an E2 (of which, at least 35 E2 enzymes are present in humans [34]), and finally transferred from the E2 to the target protein in a mechanism facilitated by one of the many hundreds of E3 Ub ligases [35]. The first ubiquitination event (mono-ubiquitination) takes place between an internal lysine (K) residue within the target protein and a diglycine motif present in the C-terminus of Ub. As Ub itself contains 7 different lysine residues, differing polyubiquitin branch structures can form depending on which lysine is the initial conjugated Ub moiety is used, which itself is determined by the combined activities of the E2 and E3 enzymes. This conjugation of mono-, multi-, and poly-Ub chains can affect a target protein's function, localization, and/or stability [36]. Typically, K48 linked Ub chains marks a protein for proteasomal-dependent degradation [37]. In addition, one study showed that Ub was capable of creating linear chains of poly-Ub through peptide bond linkage to N-terminal amino groups, which alter the functions of target proteins [38].

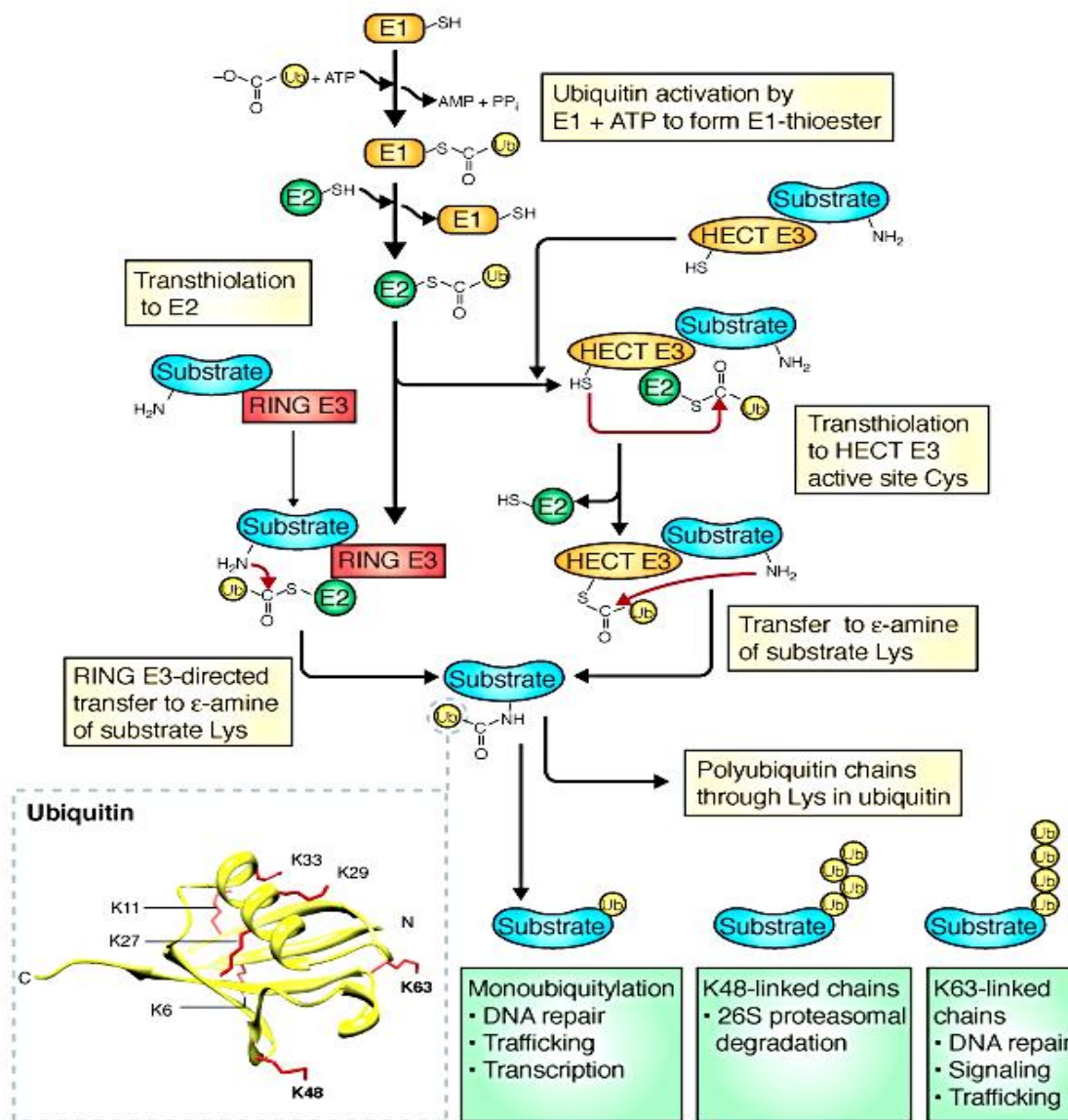


Figure 1-5. Ubiquitination is the result of three sequential events: ubiquitin-activation (E1 enzyme), ubiquitin-conjugation (E2s enzymes) and ubiquitin-protein ligation (E3s enzymes). (Adapted from [15] Metzger et al. J Cell Sci 125: 531-537; 2012).

ICP0 has the ability to synthesize or direct chains of poly-Ub in vitro and in cell culture on cellular targets, including PML [39-46]; Sp100 [39,40,46,47], CENP-A, -B, -C [48-51], DNA-PKcs [47,52,53], RNF-8 and RNF-168 [53], CD83 [54], USP7[55,56], I κ B α [57], p53 [58], IFI16 [59,60] and E2FBP1 [61], as well as certain SUMOylated targets [62]. Degradation of these targets allows HSV-1 to evade the host's intrinsic and innate responses, thereby facilitating viral replication [63]. In addition to proteasome-mediated degradation, the E3 Ub ligase activity of ICP0 was recently suggested to be linked to mechanisms that counteract the host defenses through proteasome independent processes [64], providing ICP0 yet with an additional strategy to counteract cellular antiviral defenses. Overall, the Ub ligase activity of ICP0 plays a central role in HSV-1 replication by impairing components of the hosts' intrinsic and innate antiviral responses. Intrinsic defenses are cellular factors that are always present and ready to repress viral transcription or infections [65]. Another branch of the antiviral resistance is the innate immunity, where particular molecules or patterns associated with viral replication activate a gene cascade to finally express effector proteins [66]. This activation in host gene expression leads for example to the synthesis of type I IFNs, which present a major defense mechanism against viruses [73-75].

1.2.2. Impairment of the Interferon Response

IFN proteins [67,68] are a family of cytokines, which share the ability of inducing interference of viral infections [69]. This group of cytokines is constituted by three different major components: type I, type II and type III IFNs. Within the type I IFN, encoded by intron-less genes in humans, there are 5 different types – α (composed of 13 subtypes) – β , – ϵ , – κ , and – ω (2) interferons [70]. There is only one subtype of IFN II (also known as IFN γ) which is mainly synthesized in specialized cells from the immune system such as natural killer (NK) and T lymphocytes [71]. Finally there are 3 classes of type III IFNs (also known as IL28/29): IFN- λ 1, IFN- λ 2 and IFN- λ 3 [72,73]. Of these families of IFN proteins, two members of the type 1 family (α , β) have been shown to be synthesized shortly after viral attachment or penetration and the type 1 IFN response has been shown to be essential in limiting viral infection [74-76]. Consequently the focus of our studies on type 1 IFNs will be centered in the impairment of the interferon response upon IFN β pretreatment or activation. All the interferon types or classes differ in their mechanisms of activation and their receptors on the cell surface and cytosol. Almost all nucleated cells are capable of producing and responding to type I IFN in reaction to viral infection, which is critical for the cells' antiviral resistance to HSV-1.

Viral genomes are the common feature or pattern that is recognized triggering the antiviral response. There are two different systems that independently promote IFN induction: a ubiquitous cytosolic sensor mediated by RIG-I (retinoic acid inducible gene I), MDA5 (melanoma differentiation antigen 5) and TLRs (toll like receptors) which are typically expressed in specialized surveillance cells such as macrophages and dendritic cells [77].

Although both cellular cytosolic sensors and TLRs can independently activate type I IFNs *in vivo*, they are not redundant in their functionality [66].

IFNs exert their effect through the expression of several hundreds of IFN stimulated genes (ISGs) that mediate various biological responses within the host. Some ISGs are regulated by both IFN I and II, while the others are distinctly regulated by a singular type of IFN. Many target genes are part of the intrinsic immunity other induce apoptosis and another group influences or activates components in adaptive immunity, making IFNs key regulatory mediators of the immune response. Ultimately, the stimulation of multiple IFN-induced genes by type I IFNs establishes an antiviral state and promotes immunomodulatory functions the host. Not surprisingly, the products of ISGs are often targets of viruses for the purpose of immune-evasion to ensure propagation of progeny virions [70].

The involvement of ICP0 in the impairment of an established IFN response was observed when ICP0 null mutant viruses were shown to be very sensitive to the effects of IFN- β or - α in cell culture and in a mouse model of HSV-1 latency [78,79]. The mechanism(s) by which ICP0 impairs the type 1 IFN response of the host are largely unknown. The purpose of the current study was to undertake structure/function analyses of ICP0 to identify specific domains required for this activity of ICP0, allowing us to ultimately delineate how the type 1 IFN response is disarmed by HSV through ICP0-cellular protein interactions.

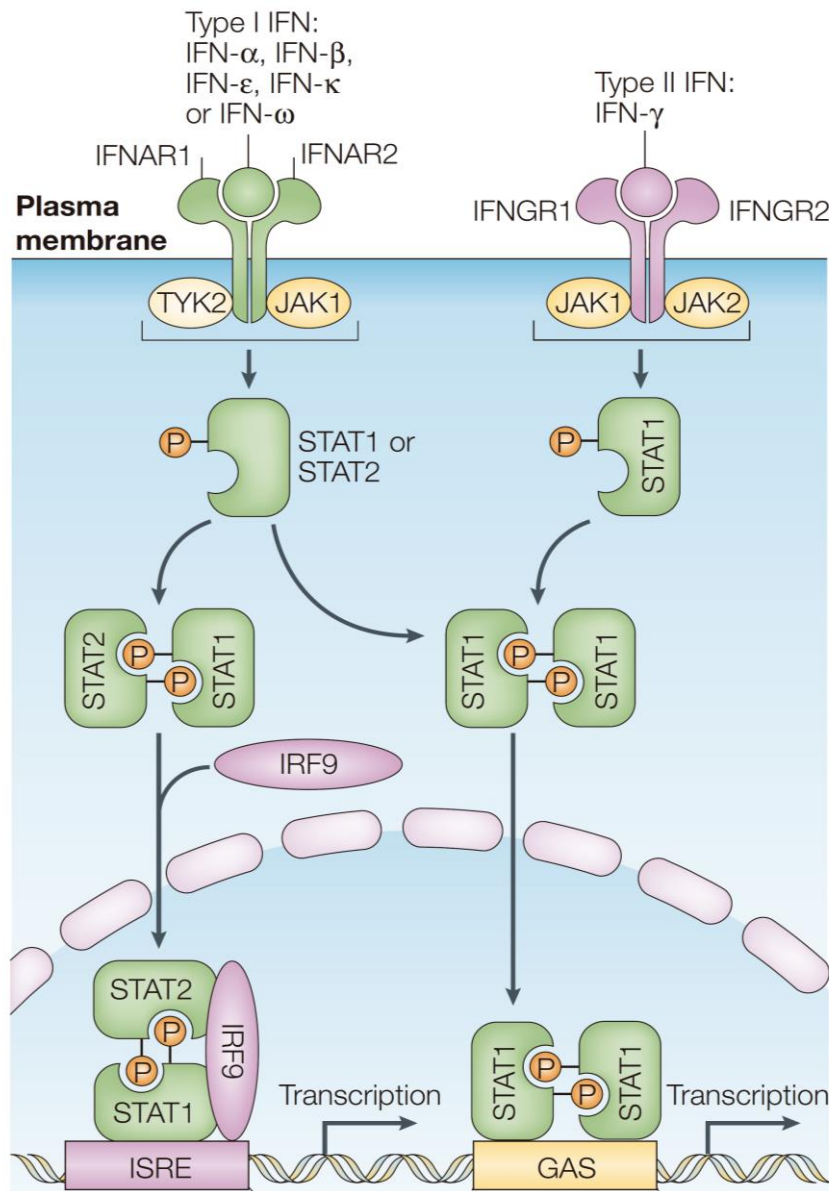


Figure 1-6. JAK-STAT pathways activation by type I and type II IFNs. All type I IFNs bind a common receptor at the surface of human cells. The type I IFN receptor (subunits, IFNAR1 and IFNAR2, associated with the Janus activated kinases (JAKs) tyrosine kinase 2 (TYK2) and JAK1, respectively). The type II IFN, IFN- γ , binds the type II IFN receptor (subunits:IFNGR1 and IFNGR2, associated with JAK1 and JAK2, respectively). Activation of the JAKs results in tyrosine phosphorylation of STAT2 and STAT1; this leads to the formation of STAT1-STAT2-IRF9 complexes (ISGF3) which translocate to the nucleus and bind IFN-stimulated response elements (ISREs) in DNA to initiate gene transcription. Both type I and type II IFNs also induce STAT1-STAT1 homodimers that translocate and bind GAS (IFN- γ -activated site) elements present in the promoter of certain ISGs, thereby initiating the transcription of these genes. Adapted from J Biol Chem. 2007 Jan 19;282(3):1757-68 [2]

1.2.3. ICP0 Disruption of ND10

Nuclear domain 10 (ND10) are protein complexes localized inside the nucleus of the cell. This organelle is involved in several cellular processes including DNA damage, apoptosis, senescence, IFN response, and protein degradation

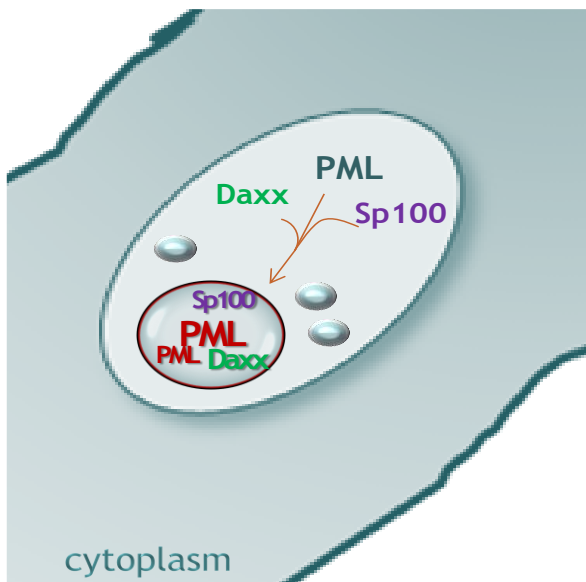


Figure 1-7. ND10 bodies are multi-protein complexes, these sub-nuclear structures that have been associated with several cellular functions among which we find the interferon response and antiviral defenses.

[80-83]. This dynamic structure is comprised of multiple cellular proteins (Figure 1-7) that are activated by HSV-1 [84] and other DNA- and RNA-containing viruses [81]. Subsequent to viral infection, ND10-associated proteins are recruited to incoming viral genomes implying a potential repressive function for some or all the components of this organelle, given that upon activation many of its constituents have shown the

ability to limit the replication of an ICP0 null HSV-1 mutant [85-87]. Among

the repressive components we find: the main component and organizer of ND10s, PML (promyelocytic leukemia) and other constitutive members Sp100 (speckled protein of 100 kDa), hDaxx (human death domain-associated protein 6), and ATRX (alpha thalassemia/mental retardation syndrome X-linked) proteins, among others. Through its E3 Ub ligase activity ICP0 can promote the disruption of ND10s by mediating either directly or indirectly the protein degradation of many ND10 components including: PML and Sp100 [39-41,46,88-91]. ICP0 in addition promotes the dissociation of hDaxx and ATRX [43]. By disrupting ND10s ICP0 appears to stimulate viral transcription by counteracting the repression of ND10 components. ICP0 has a preference to degrade small ubiquitin-like modifier (SUMO) tagged PML and Sp100 proteins [44] and reduces the overall prevalence of SUMO-tagged proteins placing ICP0 as a STUbL [44] not surprisingly, ICP0 contains the several SUMO-interacting motifs (SIMs) possesses (Figure 1-4). The depletion of various ND10 components partially complements the replication of an ICP0 null mutant, and such complementation is further enhanced if the proteins are concomitantly depleted [92].

A link between PML, Sp100, SUMO, and innate defenses has been suggested as PML and Sp100 are ISGs (reviewed in [39]), with type 1 IFNs being an important part of the innate immune response. Furthermore, SUMOylation of PML and Sp100 increases in IFN-treated cells and correlates with an increase

in the size and number of ND10s [93,94]. Overall, ND10s and other SUMOylated proteins perform to recruit cellular antiviral repressors [95], making ND10s and SUMO important players in the intrinsic response against HSV-1, which is ultimately inactivated by ICP0's E3 Ub ligase activity.

1.3. OVERALL SUMMARY AND HYPOTHESIS

Understanding mechanisms viruses use to evade the immune system and to persist within the host, is of crucial relevance in controlling, preventing and treating all the HSV-1 associated diseases. As reviewed in this dissertation ICP0 is a key viral regulatory protein that modulates the switch between lytic and latent cycles of the virus. ICP0's role in stimulating viral replication is mediated, in part, by its impairment of the host defenses.

Structure/function analyses of ICP0 described in this dissertation will enable us to begin to understand how HSV-1 ICP0 promotes the dispersion and degradation of ND10 components (Chapter 2) and dismantles the IFN response (Chapter 3). The observations from these studies can be applied to design therapeutic interventions to limit HSV-1 infections and diseases.

2. CHAPTER 2: TWO OVERLAPPING REGIONS WITHIN THE N-TERMINAL HALF OF ICP0 FACILITATE THE DEGRADATION AND DISSOCIATION OF PML AND DISSOCIATION OF Sp100 FROM ND10

Adapted from: “Two overlapping regions within the N-terminal half of the HSV-1 E3 ubiquitin ligase ICP0 facilitates the degradation and dissociation of PML and dissociation of Sp100 from ND10”

Mirna Perusina Lanfranca, Heba H. Mostafa and David J. Davido

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2.1. ABSTRACT

Herpes simplex virus type 1 (HSV-1) establishes a lifelong latent infection in sensory neurons and can reactivate from latency under stress conditions. To promote lytic infection, the virus must interact with specific cellular factors to evade the host's antiviral defenses. The HSV-1 E3 ubiquitin ligase, infected cell protein 0 (ICP0) activates transcription of viral genes, in part, by mediating the degradation of certain cellular proteins that play a role in host antiviral mechanisms. One component of the cellular defenses that ICP0 disrupts is

the sub-organelle, nuclear domain (ND) 10, by inducing the degradation and dissociation of the major organizer of ND10, promyelocytic leukemia (PML) and ND10 constituent, Sp100. Because previously identified domains in ICP0 only partially explain how it directs the degradation and dissociation of PML and Sp100, we hypothesized that additional regions within ICP0 may contribute to these activities, which in turn facilitate efficient viral replication. To test this hypothesis, we used a series of ICP0 truncation mutants and examined PML protein levels and PML and Sp100 immunofluorescence staining in human embryonic lung cells. Our results demonstrate that two overlapping regions within the central N-terminal portion of ICP0 (residues 212 to 311) promoted the dissociation and degradation of PML and dissociation of Sp100 (residues 212 to 427). In conclusion we have identified two additional regions in ICP0 involved in altering ND10 antiviral defenses in a cell culture model of HSV-1 infection.

2.2. INTRODUCTION

HSV-1 is an alpha-herpes-virus that establishes latency in humans. Infections of individuals with HSV-1 can cause cold sores, potentially blinding ocular infections, and life-threatening encephalitis. The virus has a characteristic temporal cascade of gene expression ordered into three classes: immediate-early (IE), early (E), and late (L). ICP0 (infected cell protein 0) is one of the five IE proteins that facilitates viral gene expression and impairs the host's antiviral responses to infection. Although not an essential protein, ICP0 plays a key role in the establishment of lytic infection and reactivation from latency [96-98]. ICP0 is a RING-finger containing E3 ubiquitin ligase [99]. E3 Ub ligases direct the attachment of ubiquitin molecules to target proteins, regulating their function or marking them for degradation by the proteasome. The ubiquitin-proteasome system has been characterized as a major component for cellular protein degradation, whose biological functions extend to the regulation of basic cellular processes [32,100-102]. Through its E3 ligase function, ICP0 transactivates viral gene expression from all three kinetic classes and impairs cellular intrinsic [65,103] and innate antiviral responses [78,104,105], which include chromatinization of the viral genome [106-108], inactivation of the DNA damage response [53,103,109], and the induction and establishment of the type 1 interferon response [104,105,110].

As previously mentioned, ICP0 has an important role during viral infection by counteracting host antiviral defenses. In addition to impairing the interferon response, ICP0 interferes with intrinsic defenses, which act to immediately suppress one or more steps in the HSV-1 life cycle [80,81,85,86]. A key component of the host's intrinsic resistance to viruses is PML (promyelocytic leukemia) nuclear bodies, also known as nuclear domain 10 (ND10) [111-113]. This dynamic subnuclear complex contains a variety of proteins that are recruited to these nuclear domains by PML, a major constituent of ND10 [81,114]. Other components of ND10 include the cellular proteins Sp100, hDaxx, and ATRX [43,81,113]. ND10 is associated with cellular processes that include DNA damage, protein degradation, apoptosis, senescence, and interferon signaling [80-83].

It has been hypothesized that ND10 and its constituent proteins repress transcription of the virus by enveloping viral genomes and limiting their interactions with host factors that stimulate viral transcription [44,92]. Consequently, the activation of viral gene expression by ICP0 is linked to its ability to disrupt ND10 by directing the dissociation and/or degradation of ND10 constituents such as PML and Sp100 and their SUMO-modified forms

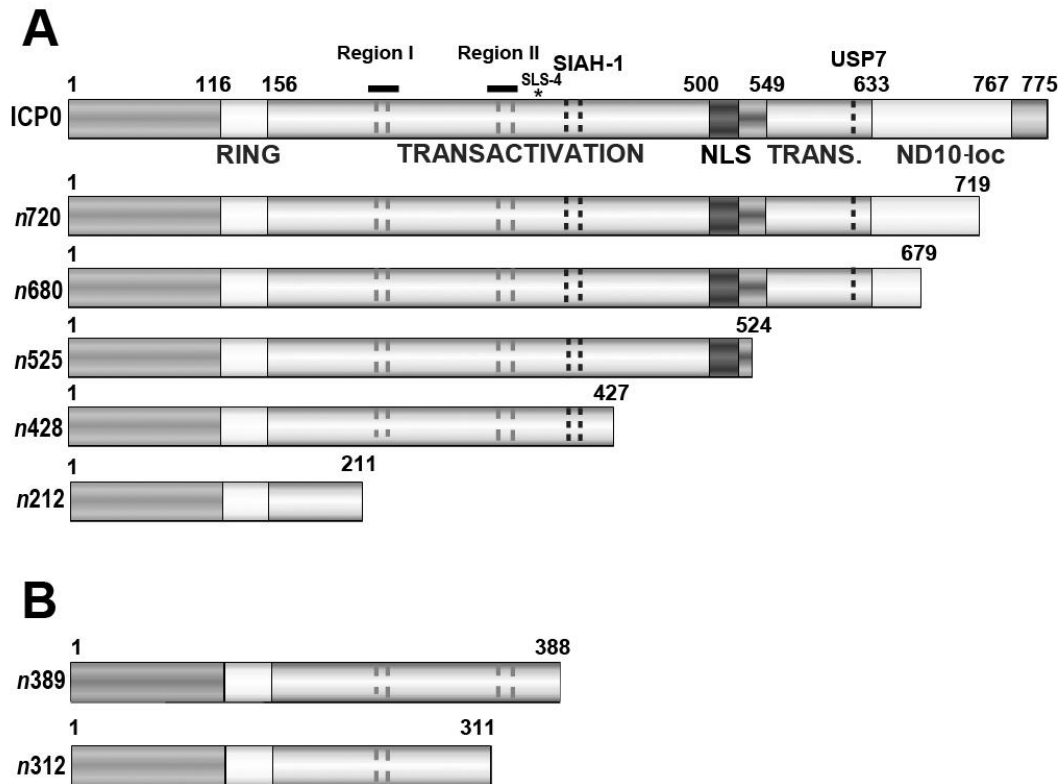


Figure 2-1. Functional domains of ICP0 and ICP0 truncation mutants.

A) Schematic of WT ICP0, expressed from WT HSV-1, and mutant forms of ICP0 expressed from the viruses *n720*, *n680*, *n525*, *n428* and *n212* are shown above; each ICP0 mutants contain a nonsense linker insertion within the ICP0 gene [12]. Selected domains and their amino acid residue boundaries are shown: a RING-finger motif, a central transactivation domain, a nuclear localization signal (NLS), a C-terminal transactivation domain (TRANS), which includes an ND10 localization sequence (ND10 – loc). Other domains include phosphorylated region I (224-232), SLS-4 (asterisk, 362-364), phosphorylated region II (365-371), a SIAH-1 binding site (400-410), and a USP7 binding site (618-638). B) Schematic of additional mutant forms of ICP0 generated as previously described [12, 10].

[39,43]. Further support that ND10 components play important roles in the function of ICP0 and the HSV-1 life cycle comes from key observations that an ICP0 null mutant can be partially complemented by cells depleted of PML, Sp100, hDaxx, or ATRX [43,85,86,92,115], with a further these enhancement in viral replication when cells are depleted for two or more of proteins [85,92].

To date, only three domains or motifs in ICP0 have been identified that mediate the disruption of ND10 via the dissociation and/or degradation of PML, Sp100, hDaxx, and/or ATRX. Specifically, the RING-finger motif (Fig. 1), which directs the E3 ubiquitin ligase activity of ICP0, is required for the dissociation of ND10 components [116]. In addition, a portion of the C-terminus (amino acids 633-767, Fig. 1) of ICP0 contains an ND10 localization domain [117,118]; this domain and the nuclear localization signal (NLS, Fig.1) of ICP0 facilitate its dissociation and degradation of PML and Sp100 and ND10-disrupting activities [46,118,119]. Recently, Cuchet-Lourenço, *et al.* have reported interactions between an N-terminal region of ICP0 (amino acids 1 to 388) with a single PML isoform (I) in two yeast hybrid assays [45], suggesting one potential mechanism by which ICP0 directs the degradation and/or dissociation of PML. Because all of these domains in ICP0 provide only a partial explanation as to how ICP0 alters PML and Sp100 levels, we hypothesized that additional regions within ICP0 likely contribute to PML and/or Sp100 dissociation/degradation, which in turn facilitates efficient viral

replication. Consequently, we performed structure-function analyses using a series of ICP0 truncation mutants (Figure 2-1) and examined PML levels by flow cytometry and western blots and the subcellular localization of PML, Sp100, and ICP0 by immunofluorescence. From our results, we conclude that the first 311 N-terminal amino acids of ICP0 mediate the dissociation and degradation of PML and the first 427 amino acids the dissociation of Sp100; residues from 212-311 and 212-427 within this N-terminal-half of ICP0 are required for both set of these activities, respectively.

2.3. MATERIALS AND METHODS

2.3.1. Cells and Viruses

HEL-299 (human embryonic lung) cells and Vero (African green monkey kidney) cells were maintained at 37°C in 5% CO₂, and cultured in either alpha minimum essential medium (αMEM) and Dulbecco's modified Eagle medium (DMEM) for Vero cells supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and 2 mM L-glutamine and 10 or 5 % fetal bovine serum (FBS), respectively. Human embryonic derived cell lines, WI-38+PML-GFP cells and L7 cells (Vero cells stably transformed with the ICP0 gene) were grown and maintained as previously described [120,121]. Wild type (Wt) HSV-1 (strain KOS), 7134 (an ICP0 null mutant), and ICP0 truncation mutants (*n*212, *n*428, *n*525, *n*680, and *n*720) were propagated as previously described [96,98]. Titers for the ICP0 mutant viruses were determined on L7 cells.

pAlter-1+ICP0 was used to create the ICP0 truncation mutants, *n*312 and *n*389, by inserting a nonsense *SpeI* linker while removing a specific restriction site (either *NruI* or *NotI*) with the pAlter site-mutagenesis kit following the manufacturer's instructions. Introduction of the mutations into the plasmid was confirmed by

DNA sequencing. Mutant viruses were generated by co-transfection of the respective mutation containing plasmid and 7134 genomic DNA at a ratio of 3:1 μ g into Vero cells plated in 60 mm dishes at 3×10^5 cells per plate. Selection of mutant viruses was performed using white/blue selection in the presence of X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) and Phenol Red for at least three subsequent rounds, and confirmed by Southern blot analysis.

2.3.2. Flow Cytometry

WI-38+PML-GFP cells or WI38-GFP cells were plated at 5×10^5 cells per well in 6-well plates. 24 h later, the cells were infected at an MOI of 5, 10, or 20 (as noted in Figure 2), with KOS or one of the ICP0 mutant viruses for 1 h at 37°C. WI-38+PML-GFP cells express the first 539 N-terminal amino acids of PML fused with GFP. Infected cells were washed 3 times with phosphate-buffered saline (PBS) to remove unabsorbed viruses and placed back into growth medium. For each time point, cells were washed three times with PBS, trypsinized, resuspended twice in 0.5% bovine serum albumin (BSA)/PBS, and fixed with 4% paraformaldehyde in 0.5% BSA/PBS. Fluorescence levels were monitored in a Beckman Flow Cytometer

(BD, Biosciences) collecting at least 20,000 events per sample. Results were analyzed using CellQuest software (BD, Biosciences).

2.3.3. Western Blots

HEL-299 cells were plated at 5×10^5 in 6-well plates. 24 h later, the cells were infected at an MOI of 5 with KOS or ICP0 mutant viruses. At 6 or 9 h post infection (hpi), the cells were washed 3 times with PBS prior to being scraped into boiling 1X Laemmli buffer containing protease inhibitors (1 $\mu\text{g}/\text{mL}$ aprotinin, 1 $\mu\text{g}/\text{mL}$ leupeptin, 1 mM phenylmethylsulfonyl fluoride), vortexed, and heated for additional 5 min at 95°C . 7.5% of each sample was resolved and using 4-12% Bis-Tris gradient gel (Invitrogen) and subsequently transferred to nitrocellulose membranes. Membranes were blocked for 1 h at room temperature with 2% nonfat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBS-T). Blots were probed for ICP0 overnight at 4°C (H11060, Santa Cruz Biotechnology) in blocking buffer. After three washes with TBS-T, membranes were probed with goat-anti-mouse IgG-horseradish peroxidase (HRP) (Jackson ImmunoResearch). After an additional three washes with TBS-T, the membranes were developed using ECL reagents (SuperSignal West Femto Chemiluminescent Substrate, ThermoFisher Scientific). The

membranes were then washed with TBS-T prior to stripping them for 30 min at 50°C in 100 mM 2-mercaptoethanol, 62.5 mM, (Tris pH 6.7), 2% SDS. Blots were again blocked as before prior to being probed overnight for PML (A301-167A, Bethyl Laboratories) and β -actin ((I-19)-R, Santa Cruz Biotechnology), both diluted in blocking buffer. After washing, the membrane was incubated with goat-anti-rabbit IgG-HRP (Jackson ImmunoResearch) and developed as before. All images were assembled using Adobe Photoshop and Adobe Illustrator.

2.3.4. Immunofluorescence

HEL cells or WI-38+PML-GFP were plated 24 h prior to infection at 50% confluence on rat collagen-coated coverslips. Cells were infected at an MOI of 5 with KOS or each mutant virus as above in α MEM. At specific time points, the coverslips were washed with PBS and fixed and permeabilized as previously described [122] Coverslips were labeled with primary antibody incubated for 1 h a tissue culture incubator at 37°C, washed three times with PBS, labeled with secondary antibody in the same manner as primary antibody, washed three times with PBS, and then mounted onto slides using ProLong antifade (Invitrogen). Primary antibodies used were: ICP0

(H11060 or rabbit polyclonal IgG PAC 3678, Pacific Immunology); PML (A301-167A, Bethyl Laboratories or sc-5621, Santa Cruz Biotechnology); Sp100 (sc-16328, Santa Cruz Biotechnology); Secondary antibodies: goat anti-mouse IgG2b Dylight 488 (Jackson ImmunoResearch); goat anti rabbit IgG Rhodamine Red-X (Jackson ImmunoResearch); donkey anti-rabbit IgG Dylight 594 (Jackson ImmunoResearch); cow anti-goat IgG Dylight 488 (Jackson ImmunoResearch). All antibodies were diluted in 0.5% FBS and 0.5% BSA/PBS. Slides were examined using a Nikon Eclipse TE-2000 U fluorescence microscope and photographed using with a CoolSNAP EZ digital camera (Photometrics) with NSI Elements Software (Nikon). Images were assembled in Adobe Photoshop and Adobe Illustrator. At least 100 cells that expressed ICP0 were examined for PML or Sp100 staining to quantify the extent Wt or each mutant form of ICP0 altered the staining of either cellular protein. Cells were categorized as either staining positive or negative for PML or Sp100. Criteria for negative staining included cells that lacked antigen expression, had half or less the intensity of staining, or had half or less the number of punctae relative to the negative controls (mock- and 7134-infected cells). In addition, we observed a minor degree of HSV Fc receptor staining, and took this staining

profile into account when we examined PML localization in HSV-1-infected cells.

2.4. RESULTS

2.4.1. ICP0 Mutants Mediate the Dissociation and Degradation of Exogenous PML

The viral IE protein, ICP0, has been shown to promote the degradation of the antiviral protein, PML [46,88,123]. To better understand how ICP0 induces the dissociation and degradation of PML from ND10, our approach was to identify new region(s) within ICP0, other than its RING finger and in its C-terminus that play a role in these functions. For these studies, we used a series of viruses in which nonsense linkers have been inserted into the ICP0 gene, giving rise to forms of ICP0 progressively truncated at its C-terminus (Figure 2-1A) [96].

To initially monitor the dissociation of PML, we examined exogenous PML-GFP fluorescence in a human embryonic cell line (PML-GFP-WI38 cells) by fluorescent microscopy at 3, 6 (Figure 2-2) and 12 hpi. After initial observations established the existence of a differential gradient ability for each ICP0 truncation mutants and the Wt virus, KOS, showing capable to promote loss of fluorescent signal from PML-GFP within the cells, we decided to use a

more precise method to quantify and compare the loss of PML-GFP promoted by each virus using flow cytometry, a method that has successfully being used by our lab to monitor ICP0's E3 ubiquitin ligase activity [120]. To examine and quantify the loss of PML-GFP fluorescence induced by KOS and each ICP0 mutant virus, cells were mock infected or infected with KOS or each ICP0 mutant, and the fluorescence signal of each sample was examined at 6 and 12 hpi (Figures 2-2A and B) [124,125]. In Figure 2-3A, histograms for mock and virally infected cells at 6 hpi were vertically aligned. The 'y' vertical line intersects the peak for the PML-GFP mock-infected sample. The 'x' vertical line intersects the KOS and *n720* peaks at 6 hpi, which showed a reduction in fluorescence compared to mock-infected cells. *n680*, *n525*, and *n428* had curves with PML-GFP levels that fell between mock- and KOS-infected samples. *n212*- and *7134*-infected samples had curves with fluorescence intensities nearly identical to mock-infected cells.

When we quantified the levels of PML-GFP fluorescence in cells, we noted that there were three statistically distinguishable groups. KOS and *n720* were very efficient at inducing the loss of PML-GFP with 12-20% levels compared to mock-infected cells, which were given a value of 100%. The next group of mutant viruses that were significantly different from KOS- and mock-infected cells was *n680*, *n525*, and *n428*. Reduced levels of PML-GFP (45-62%) were observed for *n428*, *n525*, and *n680*, although these reductions were not as

large as those resulting from infection by *n720* or KOS (Figure 2-3B). The mutant form of ICP0 encoded by *n212* was the only truncation mutant unable to significantly decrease PML-GFP fluorescence (~90% levels) for both time points, which were comparable to mock- and 7134-infected cells. (Figure 2-3B). Furthermore, even when PML-GFP-expressing cells were infected with *n212* at higher MOIs (10-20) for up to 12 h, PML-GFP signals were still largely unaffected (Figure 2-3C), suggesting that the defect in this form of ICP0 was not due to slower kinetics of PML-GFP dissociation or degradation but an inability of this the mutant to promote the efficient dissociation of PML.

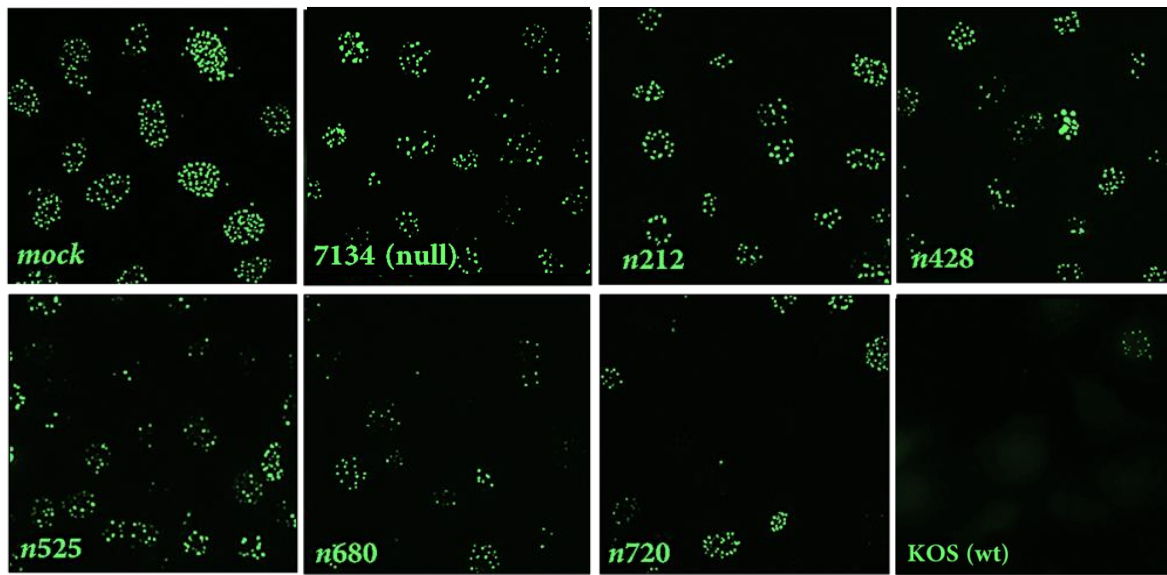


Figure 2-2. Loss of PML-GFP fluorescence by KOS and ICP0 truncation mutants by microscopy. PML-GFP-expressing cells were mock infected or infected at an MOI of 5 with KOS or each ICP0 truncation mutant viruses. At 6 hpi, cells were fixed and visualized by fluorescence microscopy at 400X magnification.

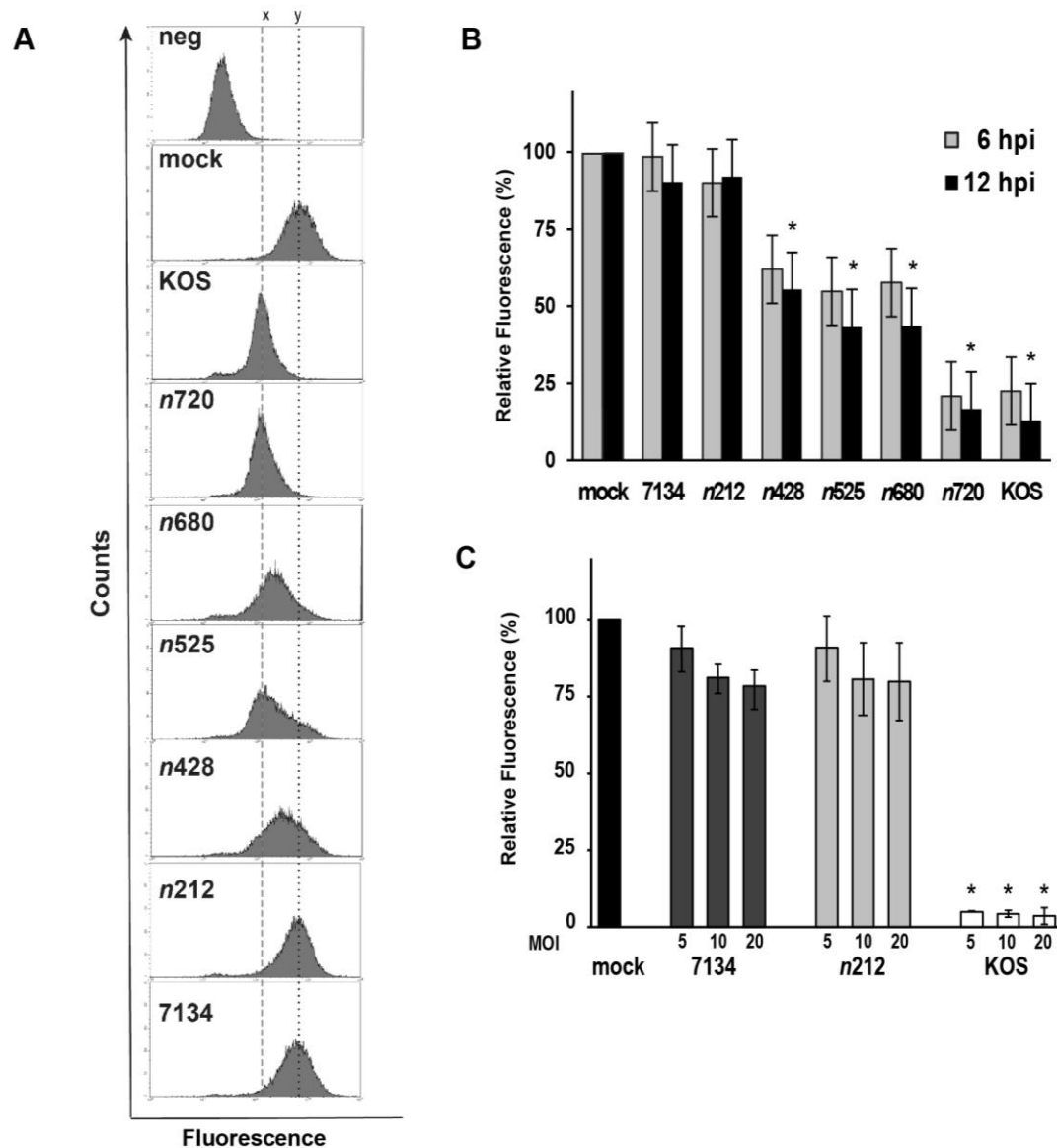
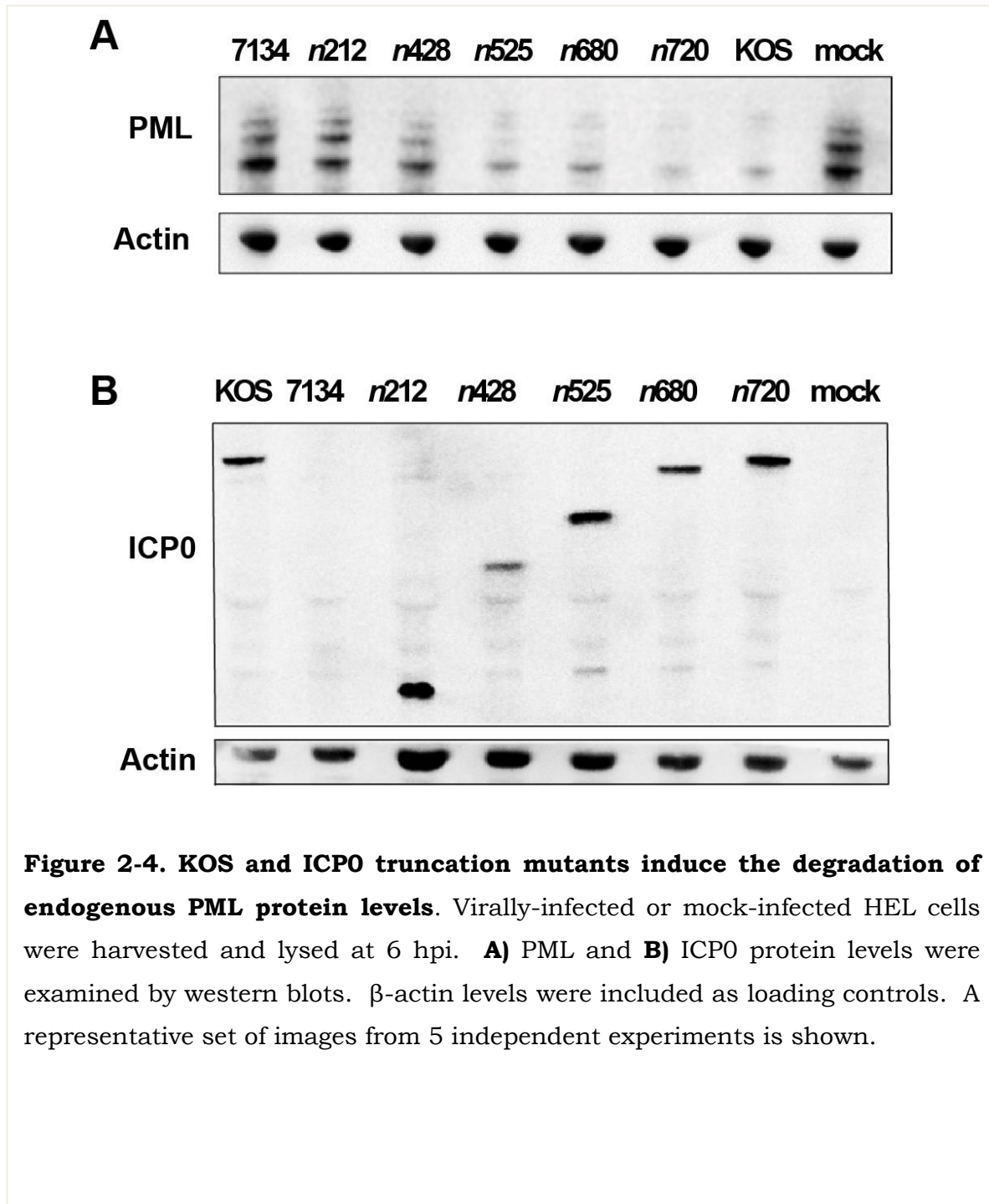


Figure 2-3. Loss of PML-GFP fluorescence by KOS and truncation mutants by flow cytometry. PML-GFP-expressing cells were mock infected or infected at an MOI of 5 with KOS or each ICP0 mutant viruses. At 6 or 12 hpi, the cells were trypsinized, fixed and analyzed by flow cytometry. **A)** Representative histograms taken from cells at 6 hpi. 20,000 events were analyzed for each histogram **B)** The MFI (mean fluorescence intensity) of each sample was used to calculate the relative fluorescence compared to the mock-infected cells (assigned the value of 100%). [Continued on next page]

6 hpi, $n \geq 13$ independent experiments; 12 hpi, $n \geq 5$ independent experiments. Data were analyzed using one-way ANOVA, Tukey's b multiple comparison post-test. Asterisks indicate significant differences ($p \leq 0.05$) relative to mock-infected cells. **C)** PML-GFP-expressing cells were mock infected or infected at an MOI of 5, 10, or 20 with KOS or ICP0 mutant viruses, n_{212} or 7134. The MFI of each sample was used to calculate the relative fluorescence compared to the mock-infected cells (assigned the value of 100%). 12 hpi, $n \geq 2$ independent experiments for each MOI. Data were analyzed using one-way ANOVA, Tukey's b multiple comparison post-test. Asterisks indicate significant differences ($p \leq 0.05$) relative to mock-infected cells.

Overall, these data show that the first 427 amino acids of ICP0 help facilitate the dissociation (and degradation) of PML-GFP from ND10 and that residues from 212 and 427 in this portion of ICP0 are required for these activities. Additionally, our results indicate that residues from 679 to 719 are necessary for ICP0 to efficiently promote the loss of PML-GFP. These data are in agreement with a previous study indicating a role for this region in the targeting of ICP0 to ND10s, facilitating the dissociation of PML from ND10 and PML's degradation [46].

2.4.2. A region of ICP0 Spanning Residues 212-427 Is Critical for the Degradation of Endogenous PML



Because we examined the effects ICP0 truncations mutants have on exogenously expressed PML, we next wanted to determine whether these mutants affected endogenous PML in a comparable manner. Consequently, we analyzed endogenous PML protein levels at 6 hpi in HEL cells (Figure 2-4). The results were generally consistent with those obtained by flow cytometry of PML-GFP (Figure 2-3A and B). Again, there was a link between the size of each mutant form of ICP0 and its ability to promote the degradation of PML. 7134- and *n212*-infected cells had PML levels similar to that of mock-infected cells. *n428* mutant form of ICP0 was capable of reducing PML protein levels, although not to same extent as the larger truncated forms of ICP0. This experiment indicated that the first 427 amino acids of ICP0 assist in the degradation of PML by 6 hpi, with the region spanning residues 212 to 427 critical for this function. Furthermore, residues from 428 to 524 are required for the efficient degradation in these assays.

2.4.3. Subcellular Localization of ICP0 Truncations Mutants

We then infected cells with KOS or each ICP0 truncation mutant for 3, 6, and 9 hpi to monitor the subcellular localization of ICP0. For Wt ICP0 expressed from KOS, our results matched those of a previous report [126] in which ICP0 localization changes over time from being primarily nuclear during the initial stages of infection (e.g., 3 hpi) to a mix of nuclear and cytoplasmic ICP0 between 3 and 6 hpi, and finally localizing predominantly to the cytoplasm at 6 hpi and later (Figure 2-5). When we examined the subcellular localization of the ICP0 truncation mutants, the mutant forms of ICP0 expressed from *n525*, *n680* and *n720* were predominantly nuclear at 6 and 9 hpi (Figure 2-5). These mutant forms possess a nuclear localization signal (NLS) (Figure 1A), and they did not localize extensively to the cytoplasm later during viral infection as observed for Wt ICP0. The *n212* and *n428* mutant form of ICP0 was found in both the nucleus and cytoplasm at 6 and 9 hpi (Figure 5). Interestingly, ICP0 expressed from *n212* and *n428* lacks ICP0's NLS, but both proteins were able to diffuse into the nucleus during infection; this localization is likely associated with their apparent molecular weights (i.e., ~35 kDa and ~58 kDa, Figure 8A), as proteins that are ≤ 60 kDa have been reported to be capable of diffusing through the nuclear pore [127]. The former two mutants, *n212* and *n428* were notably enriched in the nucleus in some cells at later

times during infection; this enrichment could be facilitated by their interaction(s) with a one or more ND10 constituents or ISG proteins.

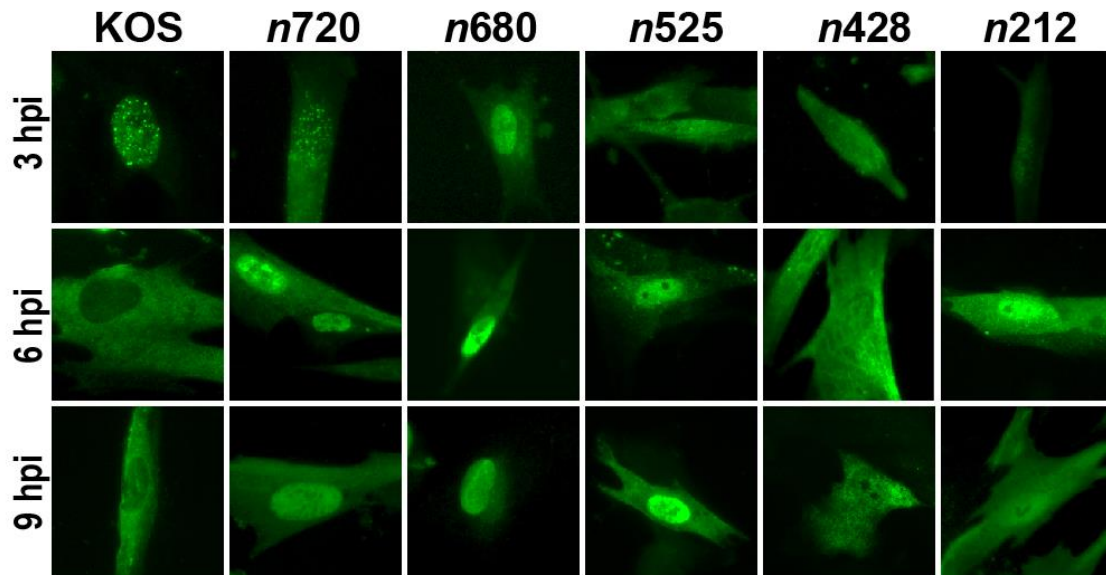


Figure 2-5. ICP0 localization in cells infected with KOS and ICP0 truncation mutants. HEL cells were infected (MOI 5) with KOS or each ICP0 truncation mutant or mock infected for 3, 6 and 9 h. Cells were fixed, permeabilized, and immunostained for ICP0 and visualized by fluorescence microscopy at 400X magnification.

2.4.4. Two N-terminal Regions of ICP0 Are Necessary for ICP0 to Dissociate PML and Sp100 from ND10

To determine how these truncated mutant forms of ICP0 affected PML localization over time, we performed immunofluorescence assays in HEL cells. Initially, cells were infected with KOS or each ICP0 truncation mutant for 3, 6 and 9 hpi to monitor the subcellular localization of ICP0 (Figure 2-6) were a time lapse of infection is depicted with accompanying schematic representation following the localization of PML and ICP0 as the infection progresses. As the infection evolves we observe PML dissociation from ND10 structures, with initial colocalization with ICP0 and further on during infection ICP0 dispersion to the cytoplasm was observed.

We then examined PML and ICP0 staining in virally-infected cells at 6 hpi because this time point resulted in >90% loss of PML staining with Wt ICP0. the staining ICP0 mutants *n720* and *n680* appeared to be similar to KOS in directing the dissociation of PML (Figure 2-7A), as $\leq 2\%$ of cells that expressed Wt ICP0 or the *n720* mutant form or the *n680* mutant form of ICP0 stained positive for PML (Figure 2-7B). Limited loss of PML staining was detected in *n525*- and *n428*-infected cells, with 8-32% of cells that expressed ICP0 positive for PML (Figure 2-7A and B). In the case of *n212* at 6 hpi, the majority (61%)

of cells that expressed this truncated form of ICP0 stained positive for PML (Figure 2-7A and B). Notably, this mutant form of ICP0 partially colocalized with PML by 9 hpi (Figure 2-8). PML staining of 7134-infected was comparable to mock-infected cells (Figure 2-7A). To exclude the possibility that the *n212* mutant was capable of dissociating PML in a delayed manner, infections with *n212* were allowed to continue for 12-24 hpi; even at these later time points, the *n212* truncation was unable to promote the further loss of PML staining. Thus, all truncation mutants were capable of promoting different degrees of PML dispersal from ND10 structures over time. Comparable results for PML staining for all of the truncation mutants were observed with two other PML antibodies (M. Perusina Lanfranca and D. J. Davido, unpublished data), demonstrating the relative specificity and reproducibility of PML staining by the Wt and mutant forms of ICP0 in this assay.

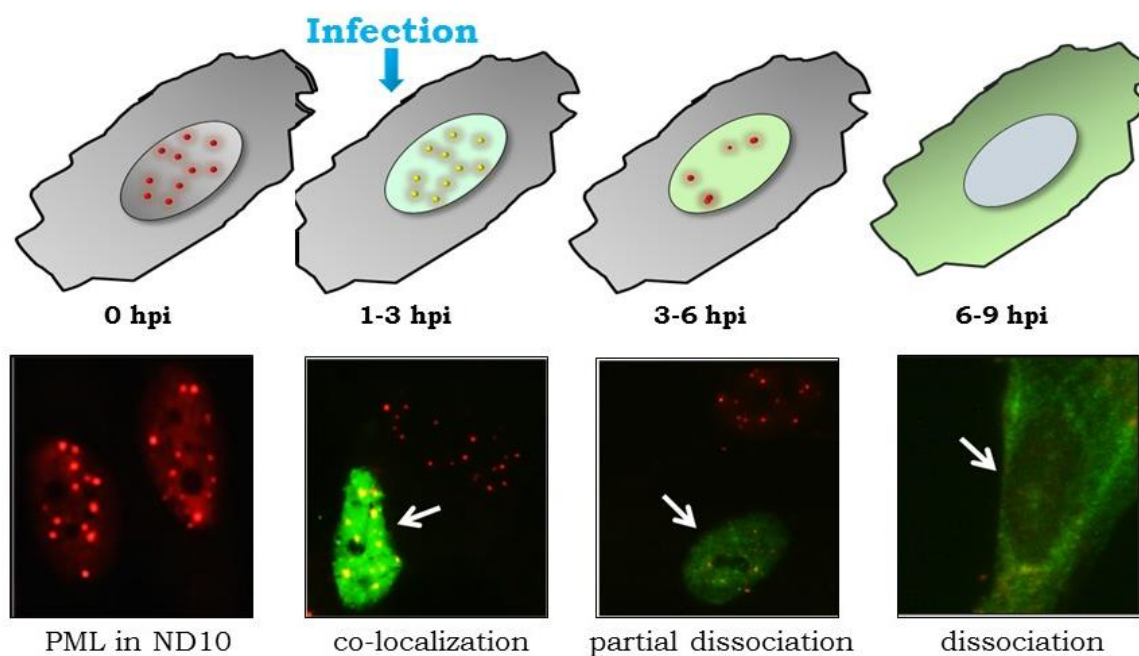
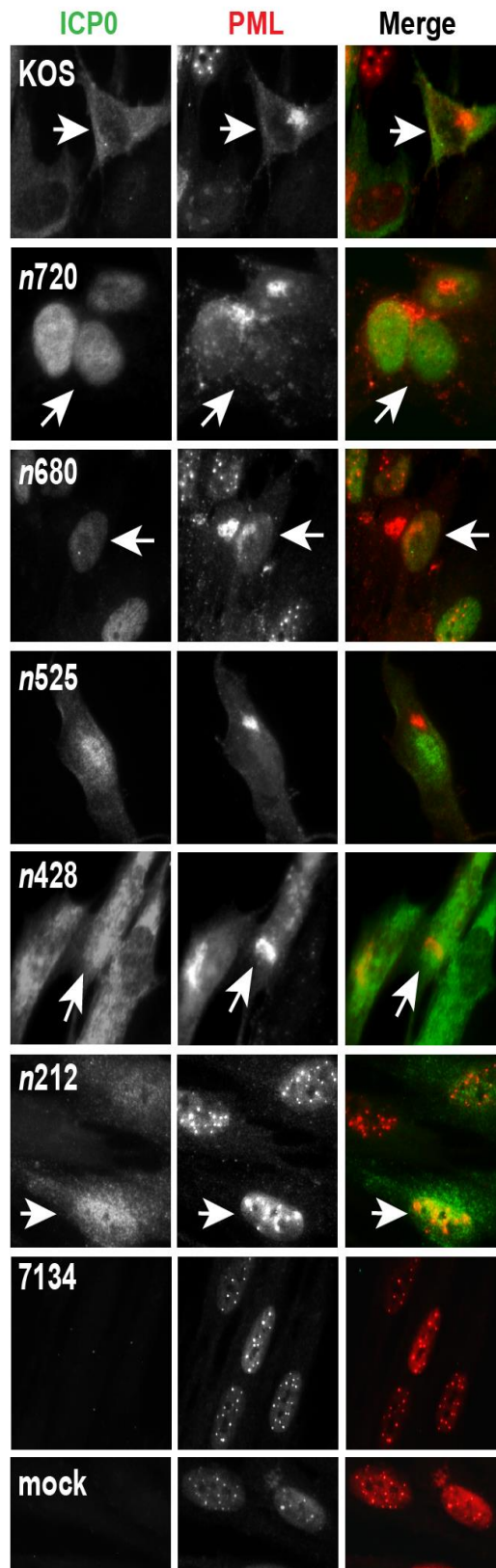


Figure 2-6. Schematic representation of PML dispersion from ND10s upon infection with Wt HSV-1. Diagram of ICP0 and PML staining with a respective immunofluorescent image shown underneath each cell. HEL cells were infected with an MOI of 5 up to 9 hpi. Cells were fix, permeabilized and stained, labelling PML in red and ICP0 in green. Arrows denote cells expressing ICP0. Co-localization between ICP0 and PML is indicated by yellow fluorescence. Cells were visualized by fluorescence microscopy at 400X magnification and images processed using Adobe Photoshop software.

A

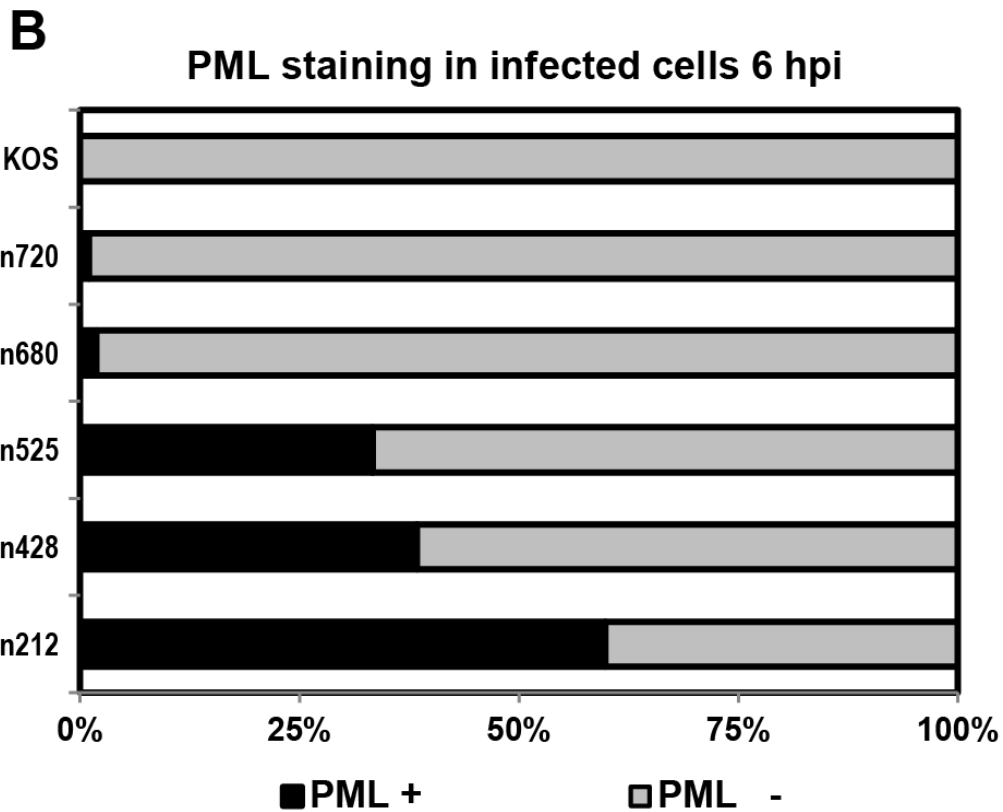


Figure 2-7. KOS and ICP0 truncation mutants mediate the loss of endogenous PML staining. **A)** HEL cells were infected at an MOI of 5 with KOS, each ICP0 truncation mutant, or 7134, or were mock infected. At 6 hpi cells were fixed, permeabilized, and immunostained with antibodies that recognize ICP0 and PML. ICP0 is labeled in green, and PML is labeled in red in the Merge. Arrows indicate an infected cell. **B)** Graph representing the percentage of ICP0-expressing cells that stained positive (+) and negative (-) for PML by immunofluorescence. For each mutant (except of 7134) at least 100 ICP0-expressing cells were analyzed with ImageJ software.

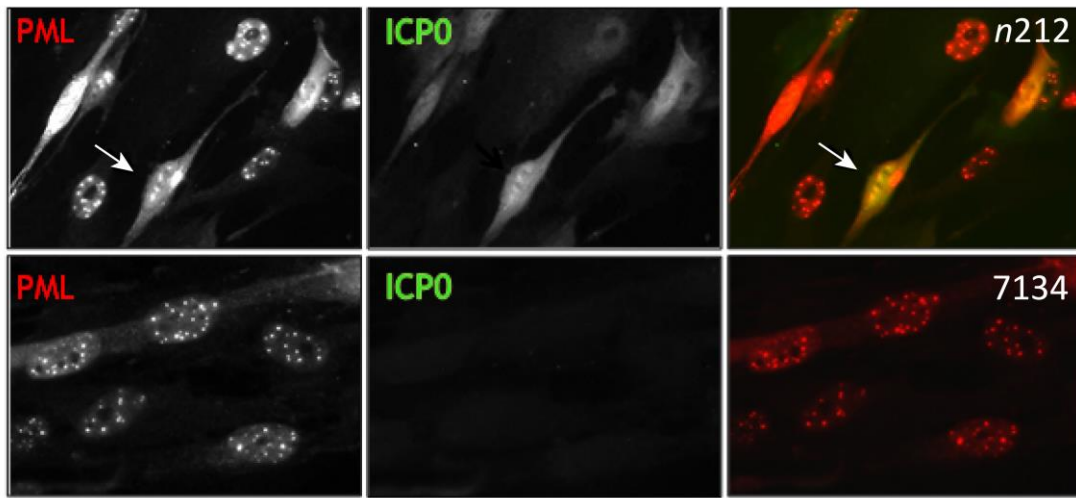
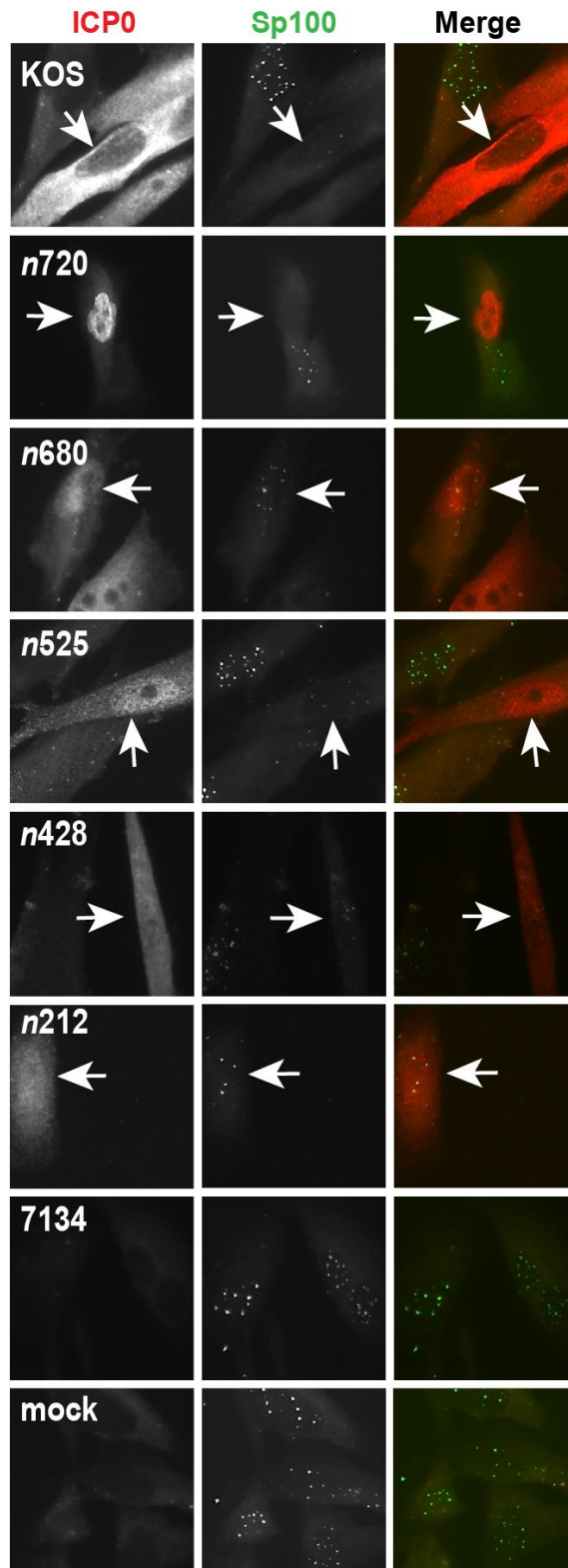


Figure 2-8. The truncation mutant *n212* induces partial dissociation of PML from ND10s by 9 hpi. HEL cells were infected at an MOI of 5 with KOS, each ICP0 truncation mutant, or 7134, or were mock infected. At 9 hpi cells were fixed, permeabilized, and immunostained with antibodies that recognize ICP0 (green) and PML (red) and colocalization as yellow in the third column.

Sp100 has been shown to stabilize ND10 by protecting PML from ICP0-mediated degradation, while promoting the repression of HSV-1 IE expression [87]. Because the ICP0 mutants showed a spectrum of PML dissociation phenotypes, we then asked how these mutant forms of ICP0 affected the staining of the Sp100. Infections were carried out as described in the PML immunofluorescence experiments, and ICP0 and Sp100 staining was monitored at 6 hpi. (Figure 2-9A). As previously reported, Wt ICP0 expressed

from KOS efficiently dispersed Sp100 from ND10 by 6 hpi (Fig. 2-9A and B) [39,42]. The staining profiles of Sp100 for the ICP0 truncation mutant viruses were similar to the results obtained with PML. *n720* and *n680* mutant forms of ICP0 were $\leq 12\%$ positive for Sp100 staining, which were in a comparable range to Wt ICP0 expressed by KOS (Fig. 2-9A and B). There was a gradual increase in Sp100 staining for *n525* and *n428*, where 23% and 29% of the *n525*- and *n428*-expressing cells, respectively, were Sp100 positive (Fig. 2-9A and B). Most cells (77%) that expressed *n212* mutant form of ICP0 contained Sp100 at 6 hpi (Fig. 2-9A and B). 7134- and mock-infected cells maintained punctate Sp100 staining (Fig. 2-9A). To confirm our results with Sp100 staining, we tried to examine Sp100 protein levels by western blot analysis using the same primary antibody from our Sp100 immunofluorescence experiments. Unfortunately, this antibody was not functional in western blot assays.

A

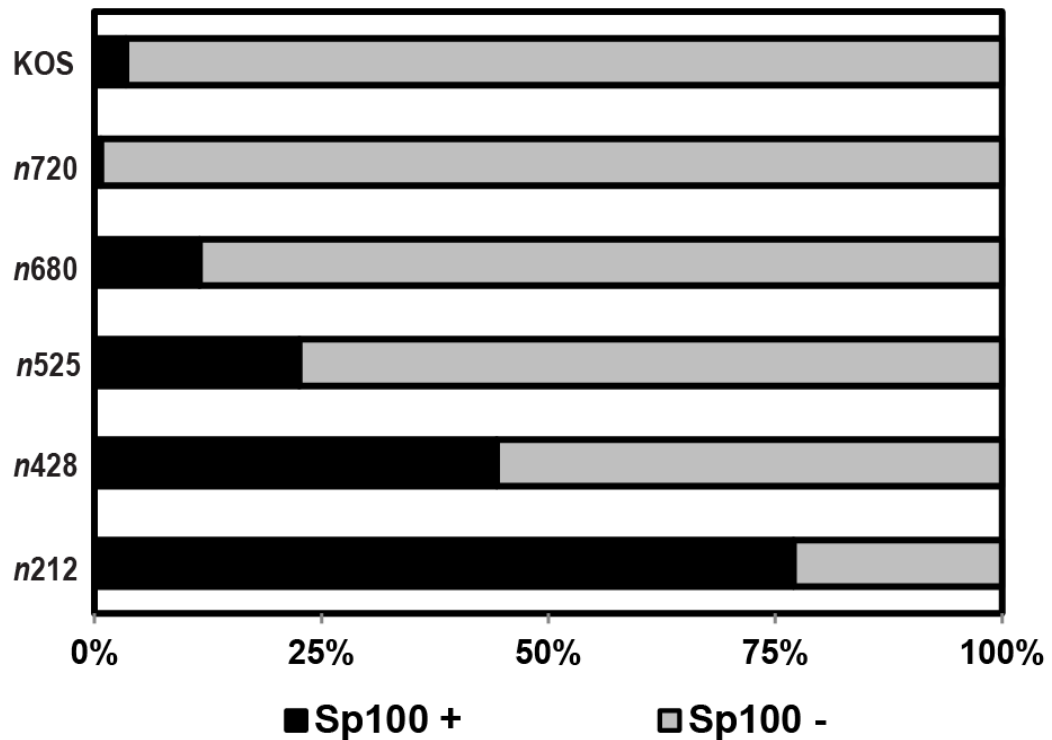
B**Sp100 staining in infected cells 6 hpi**

Figure 2-9. KOS and ICP0 truncation mutants direct the loss of endogenous Sp100 staining. **A)** HEL cells were infected at an MOI of 5 with KOS, each ICP0 truncation mutant, or 7134 or were mock infected for 6 h. Cells were subsequently fixed, permeabilized, and immunostained with antibodies that recognize ICP0 and Sp100. ICP0 is labeled in red, and Sp100 is labeled in green in the Merge. Arrows indicate an infected cell. **B)** Graph representing the percentage of ICP0-expressing cells that stained positive (+) and negative (-) for Sp100 by immunofluorescence. For each mutant (except of 7134) at least 100 ICP0-expressing cells were analyzed with ImageJ software.

2.4.5. An 100 Amino Acid N-terminal Region of ICP0 Is Key for the Degradation and Dissociation of PML from ND10

To define the sub-region from amino acids 212 to 428 of ICP0 that is necessary for directing the dissociation and degradation of PML we generated two new truncation mutants, *n312* and *n389*, which express the first 311 and 388 amino acids of ICP0, respectively (Figures 2-1B and 2-10A). Using these and other ICP0 mutants (e.g., *n428*, *n212*, and 7134), we examined endogenous PML levels by western blot in HSV-1-infected or from ND10 in HSV-1- or mock-infected HEL cells at 9 hpi. We chose to examine PML levels at 9 hpi to determine whether these mutant forms of ICP0 would be capable of directing the degradation of PML, if given additional time. As shown in Fig. 2-10A, all ICP0 linker insertion mutants we tested were capable of mediating the loss of PML, with the exception of *n212* by 9 hpi. Notably, *n312*, *n389*, and *n428* significantly directed the degradation of PML. In the case of *n428*, there appeared to be a greater degree of PML degradation at 9 hpi compared to 6 hpi (Fig. 2-4A). Thus, we have defined the region from amino acids 212 and 311 of ICP0 involved in the degradation of PML by 9 hpi. Using these ICP0 mutants, we examined the dissociation of PML and Sp100 cells that expressed the *n428*, *n389*, and *n312* mutant forms of ICP0 stained positive for PML, whereas >65% of the *n212*-expressing cells were positive for

PML staining (Figure 2-10B and C). These data largely mirrored our results for the PML western blots shown in Figure 2-10A. When Sp100 immunofluorescence was examined, there was a clear boundary between *n428* and the other truncation mutants (Figure 2-10D and E). Specifically, 22% of the cells that expressed the *n428* mutant form of ICP0 stained positive for Sp100, whereas 55-77% of *n389*-, *n312*-, and *n212*-expressing cell were positive for Sp100 (Figure 2-10E). Furthermore, our immunofluorescence experiments with *n389* and *n312* indicate that these viral mutants are not as efficient in dissociating Sp100 from ND10 compared to PML.

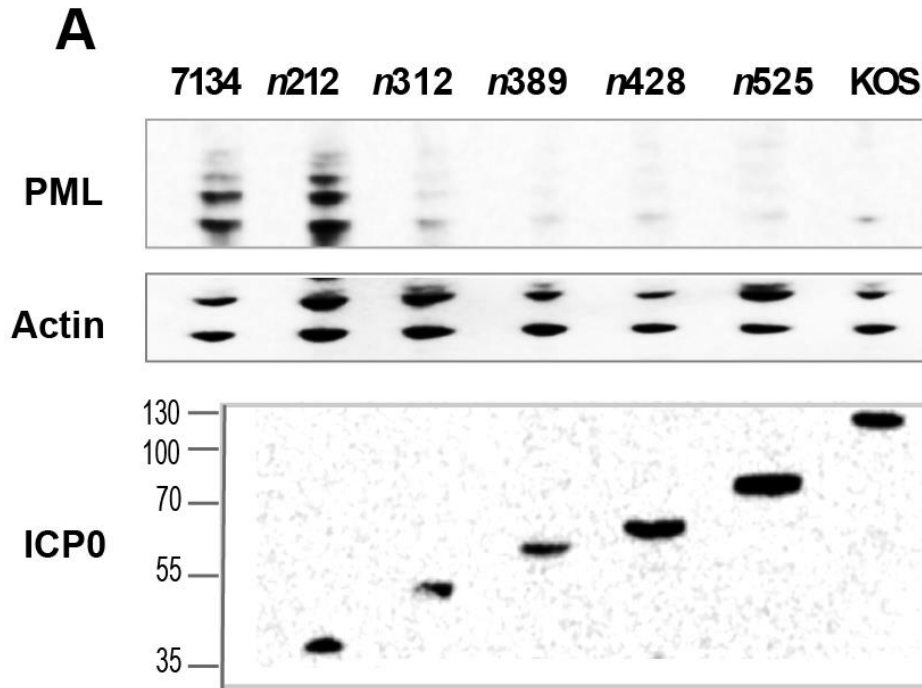
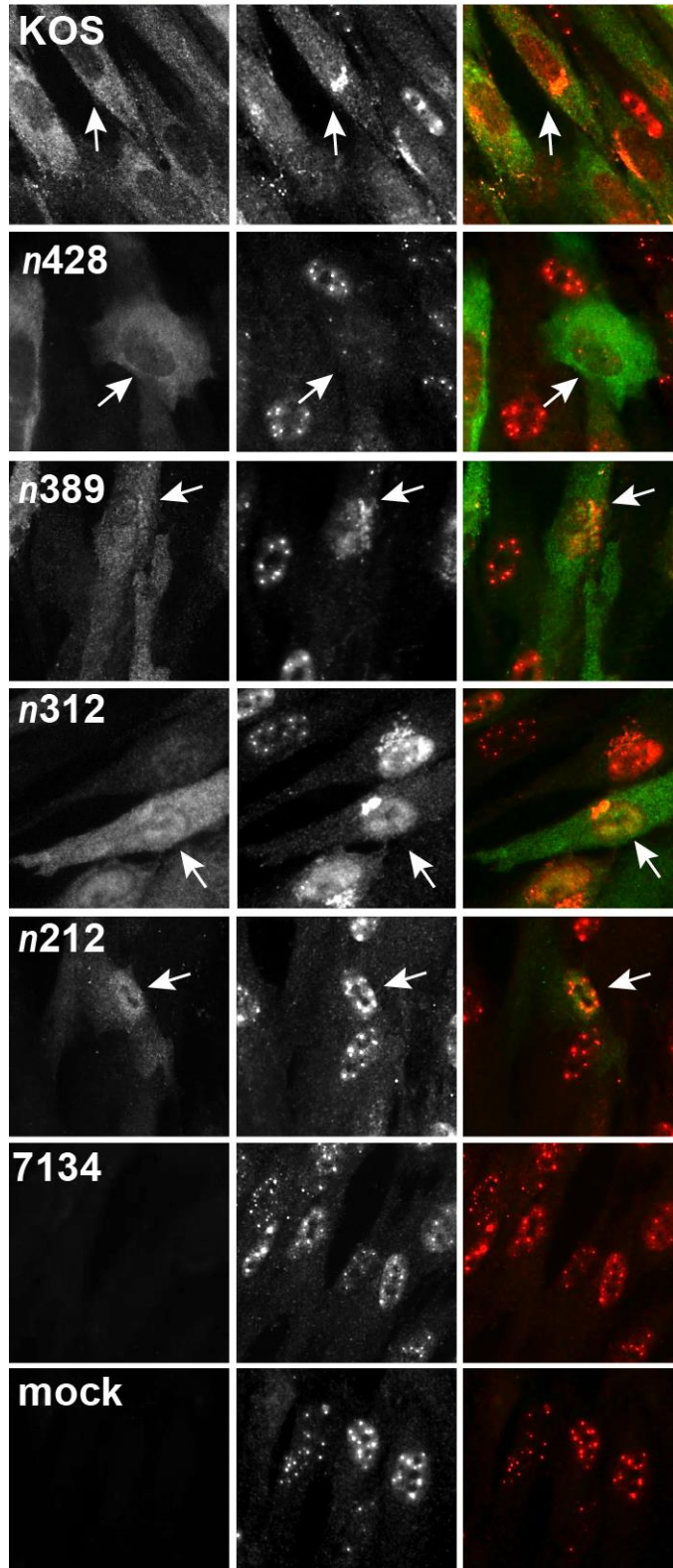
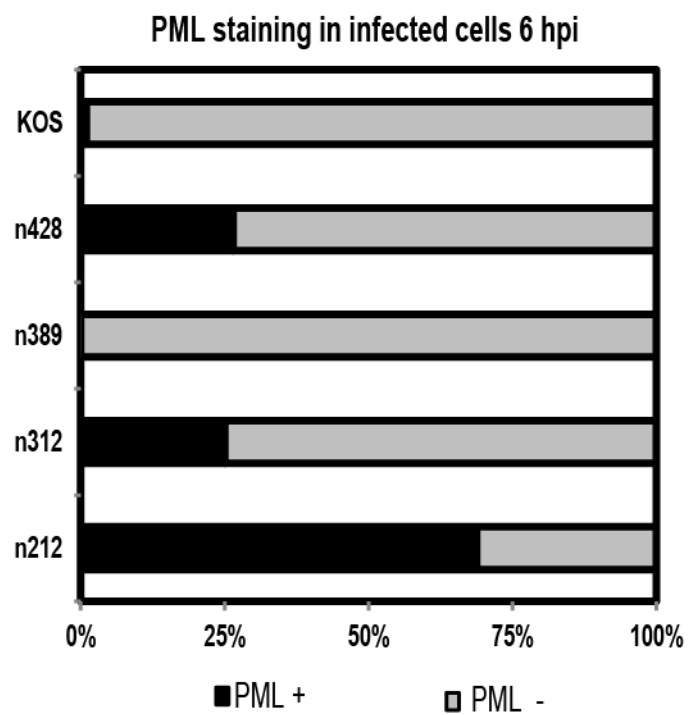
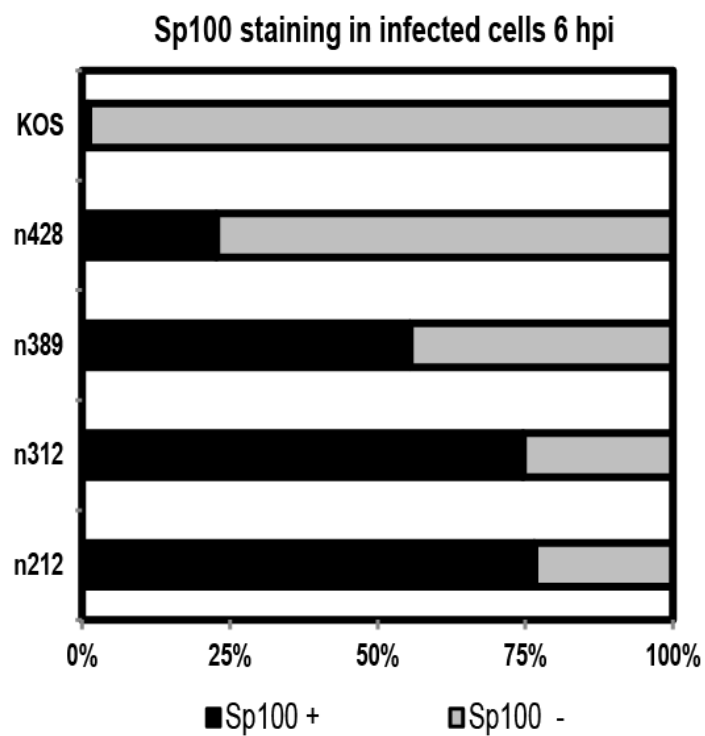
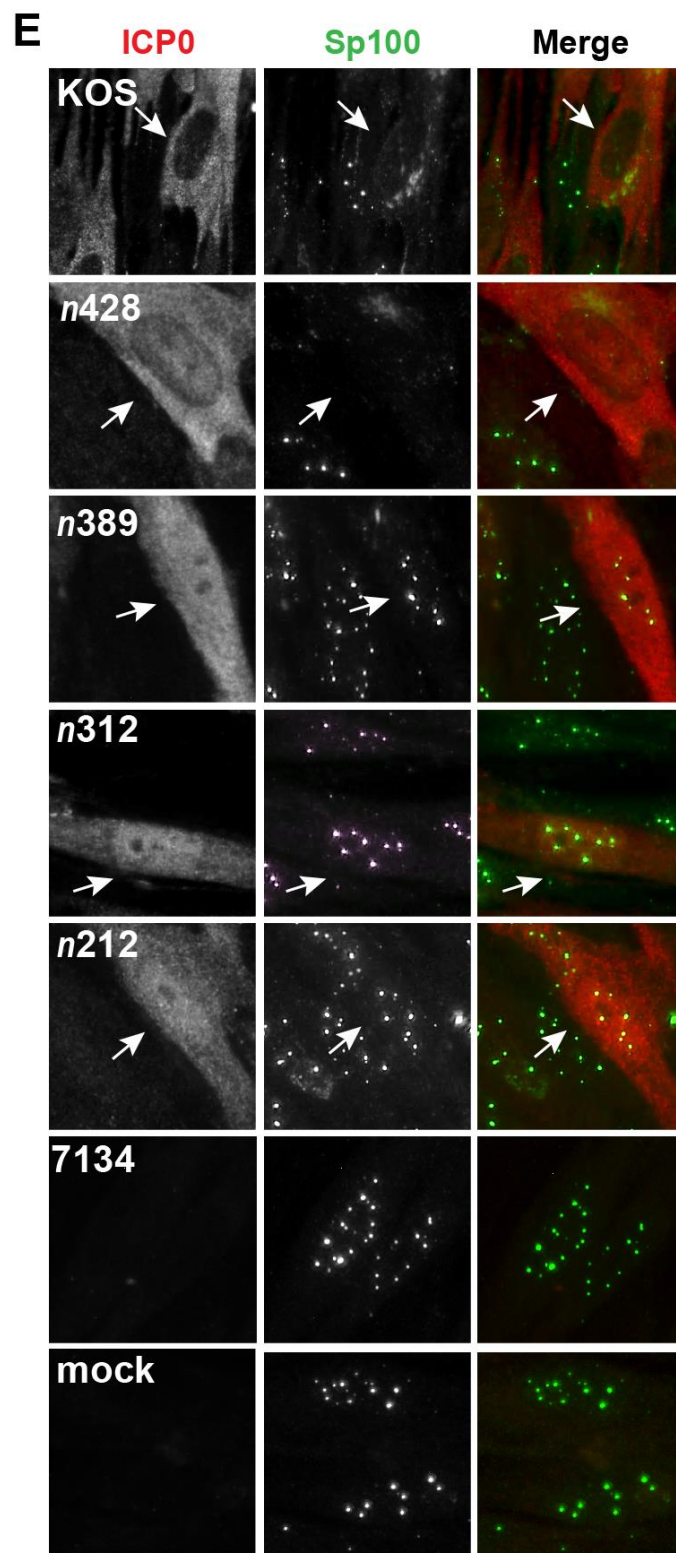


Figure 2-10. Truncation mutants *n312* and *n389* induce the degradation of endogenous PML protein levels and alter endogenous PML and Sp100 staining. A) Infected or mock-infected HEL cells were harvested and lysed at 9 hpi. PML and ICP0 protein levels were examined by western blots. β -actin levels were included as loading controls. A representative set of images from 4 independent experiments is shown. **B) - E)** HEL cells were infected at an MOI of 5 with KOS, ICP0 truncation mutants *n212*, *n312*, *n389*, *n428*, or 7134 or were mock infected for 6 h. Cells were subsequently fixed, permeabilized, and immunostained with antibodies that recognize ICP0 and either **B)** PML or **D)** Sp100. Arrows indicate an infected cell. Graph representing the percentages of ICP0-expressing cells that stained positive (+) and negative (-) for **C)** PML or **E)** Sp100 by immunofluorescence were determined for each mutant (except of 7134) by examining at least 100 ICP0-expressing cells using ImageJ software.

B**ICP0****PML****Merge**

C**D**



Summarizing our results for PML and Sp100 from Figs.2-4 through 2-10 in Table 2-1, the first 311 amino acids assists in mediating the dissociation and degradation of PML, whereas the first 427 amino acids of ICP0 direct the dissociation of Sp100 from ND10. Thus, residues from 212-311 are critical ICP0's PML dispersal and degradation activities, given that the truncated *n*212 form of ICP0 was unable to promote the degradation of PML and efficiently alter the staining of PML. Amino acids 212 to 427 are involved in the dispersal of Sp100 from ND10. Lastly, the C-terminal half of ICP0 (amino acids 428-720) facilitates the efficient dissociation of PML and Sp100.

Table 2-1. ND10 disruption and plating efficiency

virus	PML			Sp100	plating efficiency ^b
	Dissoc./loss	degradation		Dissoc./loss	
	6h	6h	9h	6h	
KOS	nearly complete	complete	complete	nearly complete	1.2
n720	nearly complete	nearly complete	complete ^a	nearly complete	13
n680	nearly complete	nearly complete	complete ^a	nearly complete	6.5
n525	nearly complete	nearly complete	complete	partial	4.5
n428	Partial	partial	nearly complete	partial	12
n389	Complete	partial ^a	nearly complete	limited	48
n312	Partial	partial ^a	nearly complete	very limited	79
n212	very limited	not evident	not evident	very limited	114
7134	not evident	not evident	not evident	not evident	310

a: unpublished data.

b: The efficiency of plating for each virus is the ratio of viral titers on L7 versus Vero cells. Values are derived from 2-4 independent experiments.

2.5. DISCUSSION

ICP0 is a multifunctional protein, and many of its functions have been linked to its E3 ubiquitin ligase activity, which requires its RING-finger domain [128]. The E3 ubiquitin ligase activity of ICP0 has been associated with the proteasomal dependent degradation of several cellular proteins, including PML [39-46]; Sp100 [39,40,46,47], CENP-A, -B, -C [48-51], DNA-PKcs [47,52,53], RNF-8 and RNF-168 [53], CD83 [54], USP7[55,56], I κ B α [57], p53 [58], IFI16 [59,60] and E2FBP1 [61], as well as certain SUMOylated targets [62]. Degradation of these targets allows HSV-1 to evade the host's intrinsic and innate responses, thereby facilitating viral replication.

In the current study, we sought to understand how ICP0 mediates the dissociation of PML and Sp100, degradation of PML, and disruption of ND10, as PML, Sp100, and ND10 play important roles in intrinsic and innate immunity [65,103]. While the RING-finger motif, NLS, and particular regions of the C-terminus of ICP0 have been shown to facilitate the dissociation and degradation of PML and Sp100 and the degradation of PML [46,123], we hypothesized that other regions or domains in ICP0 are required to impair PML and Sp100 function. This hypothesis was tested in structure-function

analyses using a series of progressively shorter ICP0 truncation mutants (Figure 2-1). We observed that there was a gradation in the ability of the mutants to mediate the dissociation of exogenous PML-GFP, the degradation of endogenous PML, and the dissociation of endogenous PML and Sp100 from ND10. Specifically, our studies show that the smallest truncation mutant, *n212*, had no or limited effects on PML-GFP, PML, and Sp100 staining and did not affect PML levels (Figures 2-3 through 2-10) by 6 or 9 hpi. This mutant form of ICP0 has been reported to possess E3 ubiquitin ligase activity *in vitro* [122]. Thus, our data indicate that the first 211 amino acids of ICP0 are partially capable of dispersing PML but do not mediate its degradation, suggesting that these two activities of ICP0 are not entirely coupled to each another. In contrast, *n428* moderately affected PML-GFP, PML, and Sp100 staining and PML levels. The remaining viral mutants, *n525*, *n680*, and *n720* were similar as or more efficient in these activities of ICP0 than *n428*, which does not contain the NLS of ICP0 (Fig. 2-1A). Thus, it is possible that the predominant nuclear localization of the *n525-n720* mutant forms of ICP0 facilitates its ND10-disrupting activities (Fig. 2-5). Notably, the PML and Sp100 staining profiles and PML levels of these ICP0 mutants largely correlate with their respective transactivation, complementation, and replication phenotypes (Table 2-1 and [96,98]). With the addition of the ICP0 mutants, *n312* and *n389*, we were able to better define the boundaries of ICP0 that result in the loss of PML protein levels and PML and Sp100 staining. Overall,

our data indicate there is a minimal region of ICP0 (amino acids 1-311) that directs the dispersal and degradation of PML, which lies within the minimal region (amino acids 1-427) of ICP0 that dissociates Sp100 from ND10; the residues from 212-311 and 212-427 are required for their respective functions.

In addition to the ICP0 structure-function analyses highlighting the importance of other motifs/regions in ICP0 (i.e., RING-finger, NLS, and C-terminus) in facilitating the dissociation and degradation of ND10 components, a recent study by Cuchet-Lourenço, *et al* indicated that ICP0 can interact with a particular isoform of PML (i.e., PML I) in co-immunoprecipitation assays [45]. These same investigators established by yeast-two hybrid assays that the N-terminal 388 amino acids of ICP0 interact with PML, and the residues from 242 to 388 are necessary for this interaction. The domain required for PML dissociation and degradation (amino acids 212-311) maps to this region of ICP0, suggesting that residues from 241-311 of ICP0's N-terminus participate in the binding of at least one isoform of PML to mediate the ubiquitination, degradation, and dissociation of PML from ND10. Also, our ICP0-Sp100 data indicate that an additional region in ICP0 (residues 212-427) is required for the dissociation of Sp100, implying that ICP0 regulates the dispersal of Sp100 by other or additional mechanisms from those required for PML.

As previously mentioned, our mapping studies identified residues from amino acids 212-311 as very relevant in playing a role for the dissociation and degradation of PML and 212-427 for the dissociation of Sp100. In addition to the aforementioned PML interaction domain, functional motifs within the larger of the two domain (212-427) of HSV-1 ICP0 (see Fig. 2-1A) include the two phosphorylated regions (Region I: 224-232 and Region II: 365-371 [129,130]), a SUMO-interacting motif (SIM) (SLS-4, residues 362-364 [44]), and a binding site for the E3 ubiquitin ligase, SIAH-1 (residues 401-410 [131]). Of these motifs, only phosphorylated Region I lies within the required PML dissociation/degradation domain of ICP0 (residues 212-311). Mutation of phosphorylated Regions I and II of ICP0 in the absence of other viral factors diminished the E3 ubiquitin ligase and/or altered the ND10-disrupting activities of ICP0 [130]. Furthermore, viral mutants containing these mutations (i.e., Phos 1 and Phos 2) were impaired for viral replication in cell culture and/or mice [130,132]. Mutation of the ICP0 SIM, SLS-4, diminished the complementation and reactivation capabilities of ICP0, especially when this mutation was expressed in truncated forms of ICP0 (residues 1-396 and 1-594) [44]. In the case of SIAH-1, its depletion increased the stability of ICP0, and an ICP0 SIAH-1 binding site mutant in HSV-2 had a modest decrease in viral replication [131]. For the PML- and Sp100-associated activities of ICP0, we speculate that these motifs likely serve collective or redundant roles and

thus their individual contributions are only apparent when expressed in the N-terminal fragment of ICP0. Future experiments will address this possibility.

Furthermore, while we have discovered that there is a minimal region of ICP0 that mediates these activities, our results also suggest that other regions/domains from amino acids 428 to 775 facilitate PML and Sp100 dispersal, PML degradation, and ND10 disruption. We propose that these additional domains in the C-terminus, besides the NLS, stabilize the binding of ICP0 to its target proteins, including PML, for ubiquitination. It is known that ICP0 can preferentially target SUMO-conjugated proteins such as PML and Sp100 for proteasomal degradation [44]. Furthermore, ICP0 contains several known SIMs, which appear to be redundant in function [44]. Thus, SUMO-SIM interactions are linked to ICP0 functions as PML and Sp100 are modified by SUMO, and PML, Sp100, and other ND10 constituents contain SIMs that are necessary for their recruitment to sites to incoming HSV-1 genomes [44,45,92,95]. Ultimately, all of these interactions contribute to ICP0's multi-faceted role in impairing the host's intrinsic antiviral response.

CONTRIBUTIONS

Conceived and Designed the Experiments: MPL, DD. **Creation of Viral Mutants:** HH, MPL; **Performed Experiments:** **Flow Cytometry:** MPL; **Western Blots:** MPL; **Immunofluorescence:** MPL **Analyzed Data:** MPL, DD; **Wrote the Paper:** MPL, DD.

3. CHAPTER 3: THE HSV-1 E3 UBIQUITIN LIGASE ICP0 REGION FROM AMINO ACIDS 1 TO 388 IS INVOLVED IN THE IMPAIRMENT OF THE HOST'S INTERFERON RESPONSE

3.1. ABSTRACT

Herpes simplex virus type I (HSV-1) is a human pathogen capable of establishing lifelong latent infections in the host and can reactivate from latency under stress. The ability of the virus to persist in its host comes from a series of activities the virus possesses to disarm distinct the immune defenses. The HSV-1 E3 Ub ligase ICP0 is a multifunctional protein that is able to promote the degradation of several cellular factors and transactivate all kinetic classes of viral genes and allows HSV-1 to replicate in the face of the host's interferon (IFN) response. Notably, ICP0 null mutant viruses have been shown to be very sensitive to the effects of an established type I IFN (e.g. IFN- α or - β) response in cell culture and in a mouse model of HSV-1 infection. Little is known about the overall mechanism(s) by which ICP0 allows HSV-1 to replicate when the type 1 IFN response has been activated. Consequently

the purpose of the current study was to identify domains in ICP0 that allow HSV-1 to be largely resistant to the antiviral effects of IFN- β .

To identify one or more domains, we used a series of ICP0 truncation mutants and performing plaque reduction, viral yields, and gene expression assays in the absence and presence of cells pretreated with IFN- β . We determine that the first 388 N-terminal amino acids of ICP0 play a role in conferring significant resistance of HSV-1 to IFN β , with residues from 212-311 mediating this resistance. We conclude that we have identified a minimal region in the N-terminus of ICP0 that contributes to the resistance of HSV-1 to an established IFN β response.

3.2. INTRODUCTION

Herpes simplex type 1 (HSV-1) is a ubiquitous pathogen that infects between 60 to 80% of the human population worldwide. Herpetic infections range from fever blisters or vesicular eruptions around the mouth and genitals to blindness and encephalitis [6,12,13]. HSV-1 infection is a severe risk for immunocompromised individuals [6,15], because the virus can spread to the brain and central nervous system (CNS), causing lethal encephalitis [16].

A hallmark of HSV-1 infections is the ability of the virus to infect and establishing a lifelong latent infection the sensory neurons of its hosts' trigeminal ganglia [133]. Latency is characterized by the absence of infectious virus while the viral genome persists in the neurons. Under conditions of stress HSV-1 can reactivate from latency leading to the production of progeny virus [6]. During productive infection, HSV-1 initiates a temporal gene expression of immediate early (IE), early (E), and late (L) genes. Among the IE proteins infected cell protein 0 (ICP0) is required for efficient lytic viral replication by stimulating all classes of viral genes [124] and regulates the switch between the lytic and latent stages of infection ICP0's E3 ubiquitin ligase activity promotes the ubiquitination and degradation of several cellular

components of the host defenses [63]. One function that ICP0 confers to HSV is resistance to antiviral factors known as type 1 IFNs.

IFNs [67,68] are a family of cytokines, capable of *interfering* with viral infections in the host [69], in part, by inducing an innate response [66]. There are three classes of IFNs in humans, in which two members of the type 1 family (i.e., α , β) are synthesized shortly after viral attachment or penetration and promote an innate immune response [74-76]. These IFNs are secreted and activate the JAK-STAT signal transduction cascade, stimulating the expression of hundreds of ISGs and a subset of proteins encoded by these genes are known to affect viral mRNA stability and processing and translation of viral mRNAs [65]. Additionally, IFNs can also stimulate the adaptive immune response of the host providing yet another mechanism by which type I IFNs limit viral replication.

Many viruses have developed countermeasures against IFNs allowing for viral persistence and transmission [70]. In the case of HSV-1, ICP0 plays an important role in resistance to IFN response. ICP0's role in modulating a pre-existing type I IFN response comes from several studies demonstrating that ICP0 null mutants are highly sensitive to the effects of type 1 IFNs, IFN- α or $-\beta$ in cell culture and in a mouse model of HSV-1 infection [78,79,134].

This deficiency in viral growth for an ICP0 null mutant can be complemented by the expression of exogenous Wt ICP0 [134].

The mechanism(s) by which ICP0 impairs a pre-existing IFN response of the host are largely unknown. To begin to address potential mechanisms, we performed structure-function analyses of ICP0 to define one or more domains required for this activity, providing possible insights into how ICP0 aids HSV-1 in disarming the inhibitory effects of IFN- β . Using replication assays in cell culture, we identified that the first 388 N-terminal amino acids of ICP0 are involved in impairing the IFN- β response, with residues from 212-388 within this N-terminal-half of ICP0 required for this impairment. Potential implications from our study are discussed.

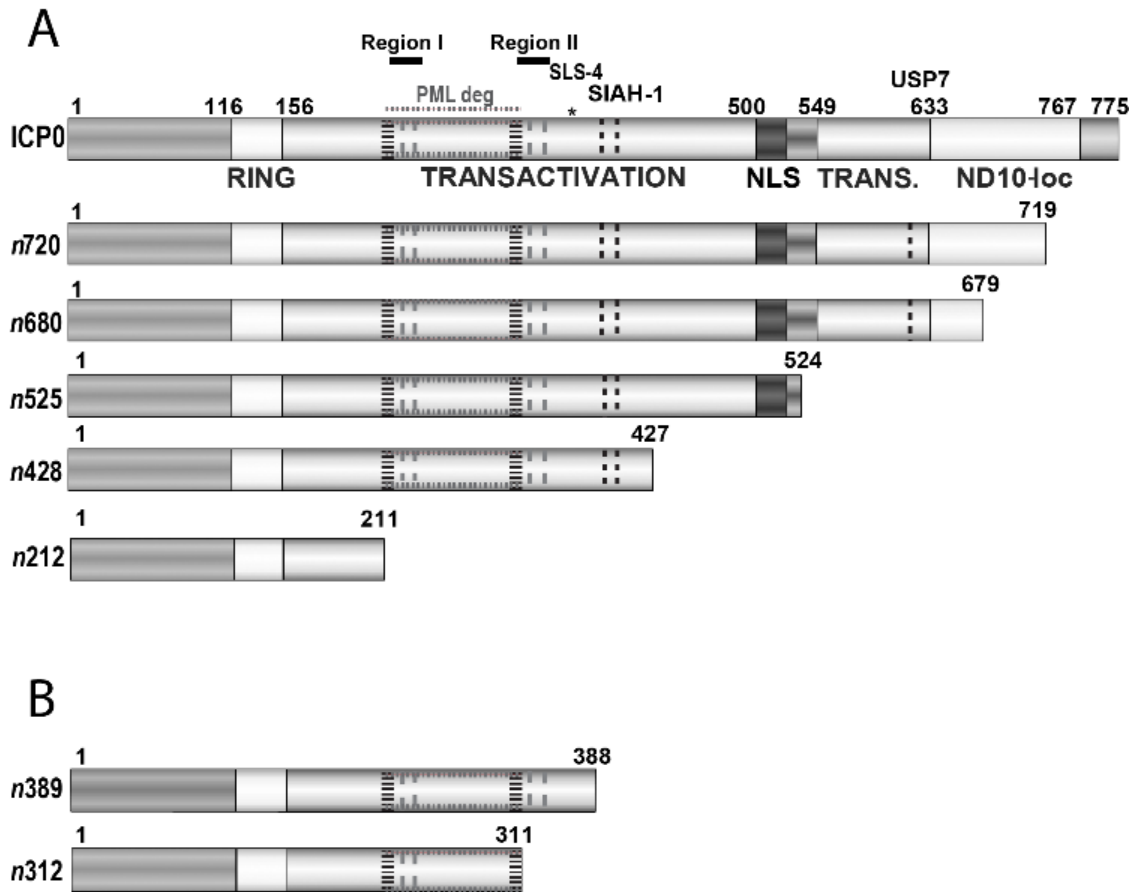


Figure 3-1. Functional domains of ICP0 and ICP0 truncation mutants.

A) Schematic of WT ICP0, expressed from WT HSV-1, and mutant forms of ICP0 expressed from the viruses *n720*, *n680*, *n525*, *n428* and *n212* are shown above; each ICP0 mutants contain a nonsense linker insertion within the ICP0 gene [12]. Selected domains and their amino acid residue boundaries are shown: a RING-finger motif, a central transactivation domain, a nuclear localization signal (NLS), a C-terminal transactivation domain (TRANS), which includes an ND10 localization sequence (ND10 – loc). Other domains include phosphorylated region I (224-232), PML degradation domain (212-312), SLS-4 (asterisk, 362-364), phosphorylated region II (365-371), a SIAH-1 binding site (400-410), and a USP7 binding site (618-638). **B)** Schematic of additional mutant forms of ICP0 generated as previously described [12, 10].

3. 3. MATERIALS AND METHODS

3.3.1. Cells and Viruses.

HEL-299 (human embryonic lung) cells, HepaRG cells (human hepatocyte cell line) and its derivatives and, Vero (African green monkey kidney) cells and its derivatives were maintained at 37°C in 5% CO₂. HEL-299 cells were cultured in alpha minimum essential medium (α MEM) containing 10% fetal bovine serum supplemented with penicillin (100 U/ml), streptomycin (100 μ g/ml), and 2 mM L-glutamine. Vero cells were cultured in Dulbecco's modified Eagle medium (DMEM) for Vero cells containing 5% fetal bovine serum supplemented with penicillin (100 U/ml), streptomycin (100 μ g/ml), and 2 mM L-glutamine. HepaRG [135] and HepaRG-shPML [92] cells were maintained in William's E media containing 10% FBS, 2 mM L-glutamine, 10 U/mL penicillin, 0.5 μ M hydrocortisone, and 5 μ g/mL insulin. L7 cells (Vero cells stably transformed with the ICP0 gene) were grown and maintained as previously described [136]. Wild type (Wt) HSV-1 (strain KOS), 7134 (an ICP0 null mutant), and ICP0 truncation mutants (*n212*, *n312*, *n389*, *n428*, *n525*, *n680*, and

n720) were propagated as previously described [96,124,137]. Viral titers for KOS were determined on Vero cells and for all ICP0 mutant viruses were determined on L7 cells.

3.3.2. Plaque Reduction Assays.

HEL-299, Hepa-RG, Hepa-RG shPML, Vero, or L7 cells were plated in 24-well plates at 1×10^5 cells/well. The next day cells were untreated or treated with 1000 U/ml of human IFN- β (AbD Serotec) in their respective medium. After 15 h of IFN-treatment, cells were infected with 10-fold serial dilutions of the respective Wt HSV-1 and ICP0 mutant viruses. After a 1 h incubation at 37°C, cells were overlaid with cell culture medium containing 0.5% methylcellulose with or without IFN- β (1000 U/ml). After 3 days post infection, cultures were washed with PBS and fixed with 3.7% formaldehyde, probed 1 h, at RT (room temperature) with rabbit anti-HSV-1 polyclonal antibody and another hour with a horseradish peroxidase conjugated anti-rabbit (Jackson ImmunoResearch). Plaques were visualized with Vector Red substrate (Vector Labs). Stained plates were scanned with a scanner (Cannon), and plaque numbers and image processing were done using Image J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA,

<http://imagej.nih.gov/ij/>, 1997-2014) and Adobe Photoshop software. Ratio was calculated as the titer of the viral stock (PFU/ml) in the absence of IFN- β over the titer of each viral stock in the presence of IFN- β .

3.3.3. Viral Growth Yield Assays.

Hepa-RG cells were plated in 12-well plates at 1×10^5 cells/well, and the next day cells were either mock-treated or not with 1000 U/ml of human IFN- β . 15 h prior to infection. Cells were infected with HSV-1 or ICP0 mutants at an MOI of 1 for 1h, and acid wash treated to inactivate unabsorbed viruses, adding back medium in the absence or presence of 1000 U/ml of human IFN- β . After 24 h of infection, samples were harvested and frozen at -80°C . The titer of each viral sample was determined on Vero (KOS) or L7 (ICP0 truncation and deletion mutants) cells, respectively. Statistical analysis was done using Paired *t* test ($p \leq 0.05$).

3.3.4. RT qPCR

HEL-299, HepaRG and HepaRGshPML cells were plated at 1×10^5 cells per well. Next day cells were treated or not treated with human IFN- β at 1000 U/mL. After three PBS washes cells were harvested

in Trizol (Invitrogen), and cDNA synthesized with iScript cDNA synthesis kit (Bio-Rad). For each sample, real time PCR was performed using FastStart SYBR green master (Rox) (Roche) in a StepOnePlus Real-Time PCR System (Applied Biosystems). Transcripts were amplified using the following primer sets:

hTBP (5'-TGCACAGGAGCCAAGAGTGAA-3' and 5'-CACATCACAGCTCCCCACCA-3'),

ISG15 (5'-GGTGGACAAATGCGACGAAC-3' and 5'-ATGCTGGTGGAGGCCCTTAG-3') and

PML (5'-AGGAAGTGCAGCCAGACCCAGTG-3' and 5'-TGAGACTGCCTTGGAGGTGCTGG-3').

hTBP levels were used to normalize other transcript levels.

3.4. RESULTS

3.4.1. A Region Between Amino Acids 212 and 427 within the N-terminus of ICP0 Is Involved in the Impairment of the IFN Response

As previously mentioned, ICP0 is crucial for the ability of HSV-1 to overcome a pre-induced type 1 IFN response. Using the ICP0 truncation mutants in mentioned in the previous chapter along with Wt HSV-1 and an ICP0 null mutant (7134), we performed plaque reduction assays in 3 cell types in the absence and presence of IFN- β . Of the cell types examined, we used Vero (African green monkey kidney) cells, which are responsive to IFN treatment but unable to synthesize it [138]. Additionally, we chose a primary cell strain, human embryonic lung (HEL) 299 cells, for these experiments, as HEL cells are capable of responding to and synthesizing type 1 IFNs induced by viral infection [126,139]. Lastly, a Vero complementing cell line (L7 cells), which contains the ICP0 gene, was included in this study to confirm that alterations in ICP0 gene were responsible for the plaquing phenotypes we observed with our ICP0 mutants.

When we evaluated the plaque reduction assay results performed in the presence IFN- β , in all three cell lines, we noticed that there was a reduction in plaque size for all viruses tested compared to the absence of IFN- β , with the exception of 7134 and *n212*, which will be discussed later. These results indicate that IFN- β impacts the HSV-1's ability to spread from cell-to-cell, whether ICP0 or a mutant form of the protein is expressed by the virus.

We then compared the plaquing efficiencies of each virus on the 3 cell lines by taking the ratio of viral titers in the absence of IFN- β versus in the presence of IFN- β . Consequently, the higher the ratio the more difficult it is for a virus to plaque on IFN- β -treated cells. For 7134 and *n212*, plaques could not be detected with these viruses upon the addition of IFN- β . In this instance, the lowest dilution of virus tested was given the value of 1 to estimate the level of its impairment.

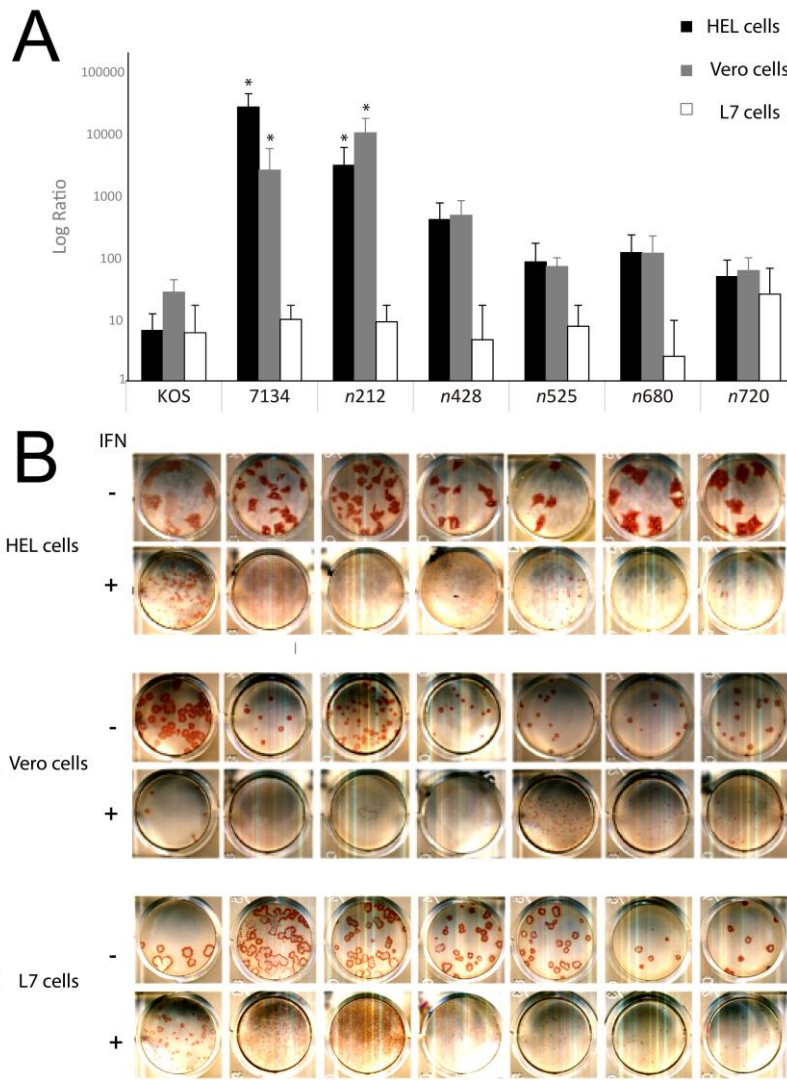


Figure 3-2. Wt HSV-1, ICP0 null, and ICP0 C-terminal truncation mutants in plaque reduction assays with the presence and absence of IFN- β . HEL, Vero and L7 cells were plated in 24-well plates, treated or not with 1000 U/ml of human IFN- β in their respective medium. After 15 hours of IFN-treatment, cells were infected with 10-fold serial dilutions of the respective ICP0 Wt (KOS), null (7134) and C-terminus truncation mutant viruses. 3 dpi cells were fixed and stained by Immunohistochemistry with a polyclonal antibody anti-HSV-1. **A)** Titers were determined as PFU/ml in -/+ IFN for KOS, 7134 and C-terminal truncation mutants. Asterisks indicate that plaques were not identified for these viruses at the lowest dilution tested with IFN- β and were given the value of 1 for estimating this ratio. **B)** Images of infected wells showing immunohistochemically stained plaques of each virus are shown for all three cell types in the -/+ IFN- β . n: 4 – 14 for **A)** and **B)**.

When we examined the plaquing of Wt HSV-1 and ICP0 mutants in HEL and Vero cells, we were unable to detect plaques in the presence of IFN- β for 7134 and the smallest ICP0 truncation mutant, *n212* [125,140]. Previous reports have indicated that ICP0 *n212* is a null mutant. However, we were able to detect the expression of this protein during infection (Figures 2-5, 2-10 and [91]), this mutant form of ICP0 appears to possess some E3 Ub ligase activity *in vitro* [122], and it can colocalize and disperse PML from ND10, albeit in a limited manner [91]. Based on these results, we do not consider *n212* to be a true ICP0 null mutant. Nevertheless, these experiments showed that *n212* is as severely compromised as ICP0 null mutant in IFN- β -treated HEL and Vero cells (Figure 3-2 and 3-3). The rest of the truncation mutants exhibited a decrease in their sensitivity to IFN- β that approximately correlated with their size (summarized in Table 3-1). In the two cell types, the ratio for *n428* ranged from 20-80 fold relative to KOS (Wt HSV-1). Reductions in *n525*, *n680*, and *n720* ranged from 10-60 fold compared to KOS. All truncation mutants were capable of plaquing in the presence of IFN- β with the exception of *n212*, suggesting that the region between amino acids 212 and 427 of ICP0 plays a

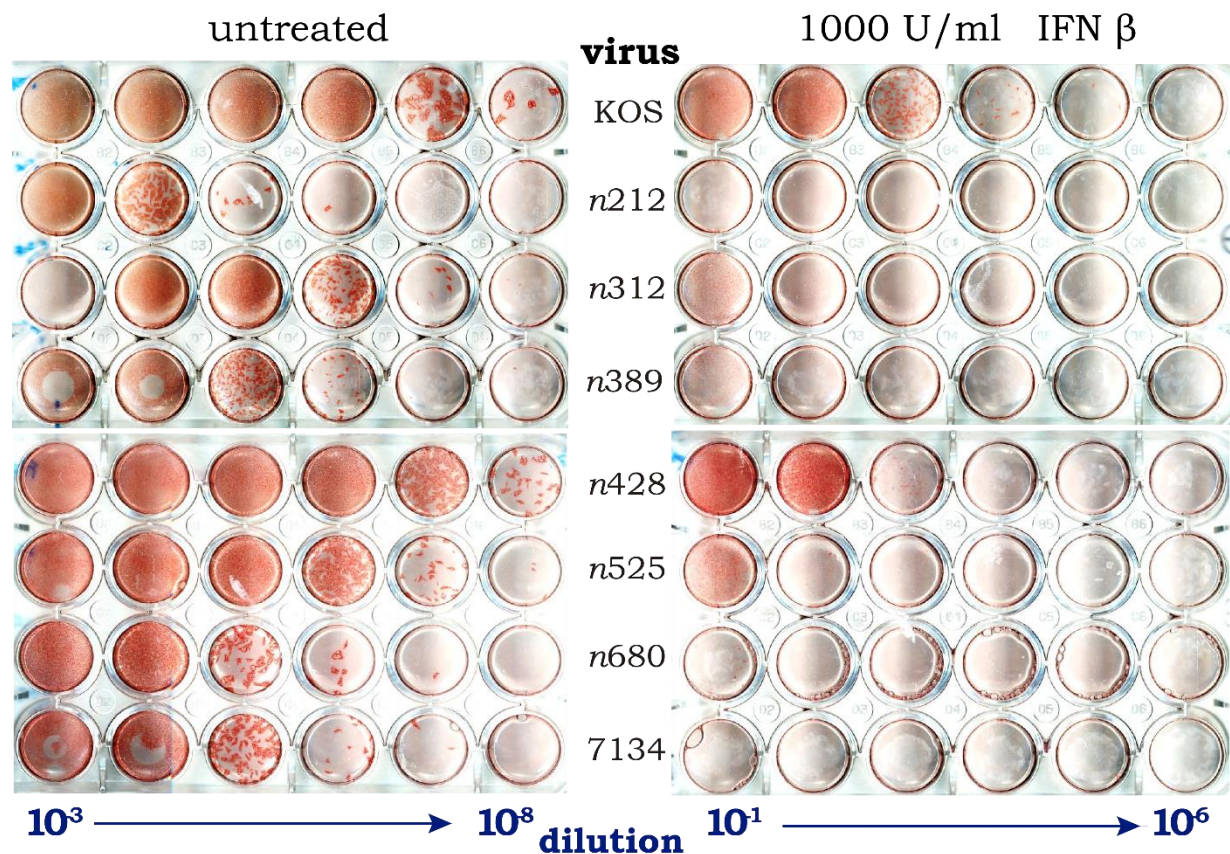


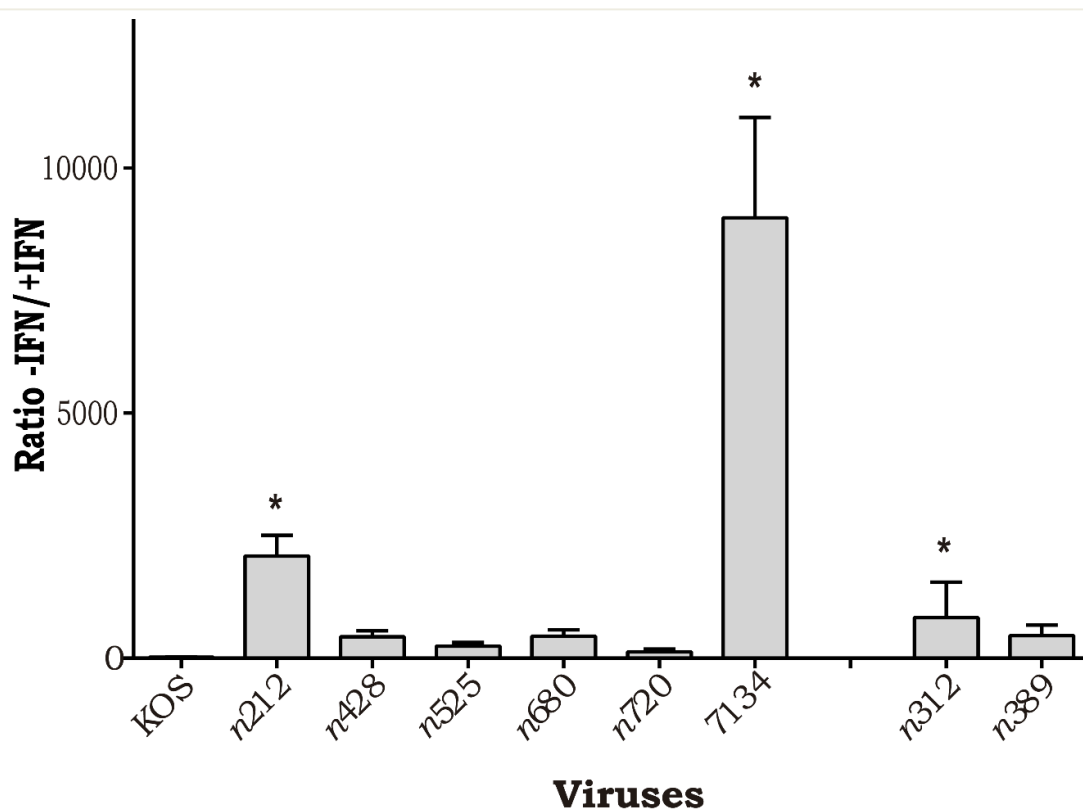
Figure 3-3. Plaque reduction assay with KOS, 7134 and ICP0 truncation mutants in HEL cells. HEL cells were plated in 24-well plates, treat or not with 1000 U/ml of human IFN-β in the respective media. After 15 hours of IFN-treatment, cells were infected with 10-fold serial dilutions as indicated, of the respective ICP0 Wt (KOS), and ICP0 null (7134) and truncation mutant viruses. 3 dpi cells were fixed and stained by Immunohistochemistry with a polyclonal antibody HSV-1. Viral plaques are shown in red.

defects in plaquing induced by IFN- β were overcome in L7 cells, indicating the effects we observed for all ICP0 mutants tested could be complemented by Wt ICP0 (Fig. 3-2A and B).

Once we defined the region from residues 212 to 427 of ICP0 as facilitating HSV-1 replication when induced with IFN- β , we sought to better map region(s) in this portion of ICP0 responsible for this phenotype. As a result, two additional mutant viruses with stop codon linker insertions were generated as previously described [91] and included in our plaque reduction assay. These mutants, *n312* and *n389* (Figure 3-1), were tested under the same conditions in HEL cells with the majority of viruses used in our previous experiments (Fig. 3-2). An example of a plaque reduction assays with these viruses is shown in Figure 3-3.

Table 3-1. Wt HSV-1 and Truncation Mutants' Relative Sensitivity to IFN- β

<u>Viruses</u>	<u>Sensitivity to IFN-β</u>
7134, <i>n</i>212 & <i>n</i>312	+++++
<i>n</i>389	++++
<i>n</i>428	+++
<i>n</i>525, <i>n</i>680 & <i>n</i>720	++
KOS	+



Means	21	≥2089	436	245	449	130	≥8985		≥830	460
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Figure 3-4. Plaque reduction assay in HEL with KOS, 7134 and ICPO truncation including n312 and n389. HEL cells were infected and treated as in Fig. 3-4 with 1000 U/ml of human IFN- β . Ratio calculated corresponds to $n \geq 4$ experiments, with error bars representing the standard errors of the means. Asterisks indicate that plaques were not identified for these viruses at the lowest dilution tested with IFN- β and were given the value of 1 for estimating this ratio.

For these experiments, *n312* was as sensitive as *n212* and the ICP0 null mutant to IFN- β , such that we were essentially unable to detect plaques. *n389* was impaired for plaquing upon the addition of IFN- β to basically the same extent as *n428*. From this new set of experiments we would classify *n312* as being very sensitive to IFN group and *n389* as being only moderately sensitive. With this data, we have defined the region from residues 1-388 of ICP0 as important for HSV-1 to impair a pre-induced IFN- β state.

3.4.2. ICP0 Viral Growth Yields in the Presence of INF- β Indicate that the Region Spanning from Residues 1 to 388 Is Responsible for ICP0's Impairment of the Interferon Response

To determine if the phenotypes observed in our IFN- β plaque reduction assays correlated with reductions in viral replication, we performed viral growth assays in the absence and presence of IFN- β . Using the same viruses as in the plaque reduction assay, HEL or HepaRG cells were infected at an MOI of 1. We included HepaRG cells in these experiments as there is a high requirement for ICP0 on HSV-1 replication in these cells, and they are responsive to IFN- β [135]. When compared side by side, both HEL and HepaRG

cells respond to exogenous IFN- β by stimulating the expression of two typical ISGs, ISG-15 and -56 (Figure 3-5).

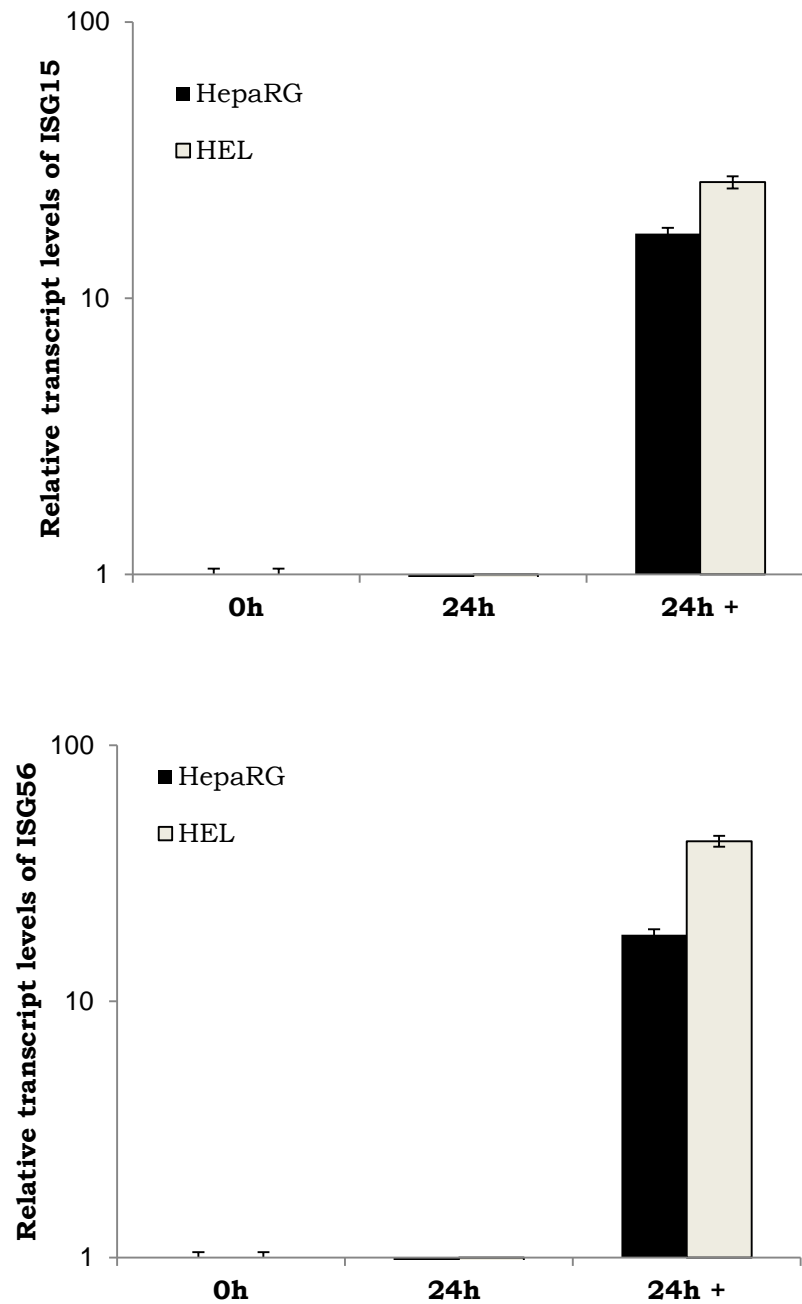
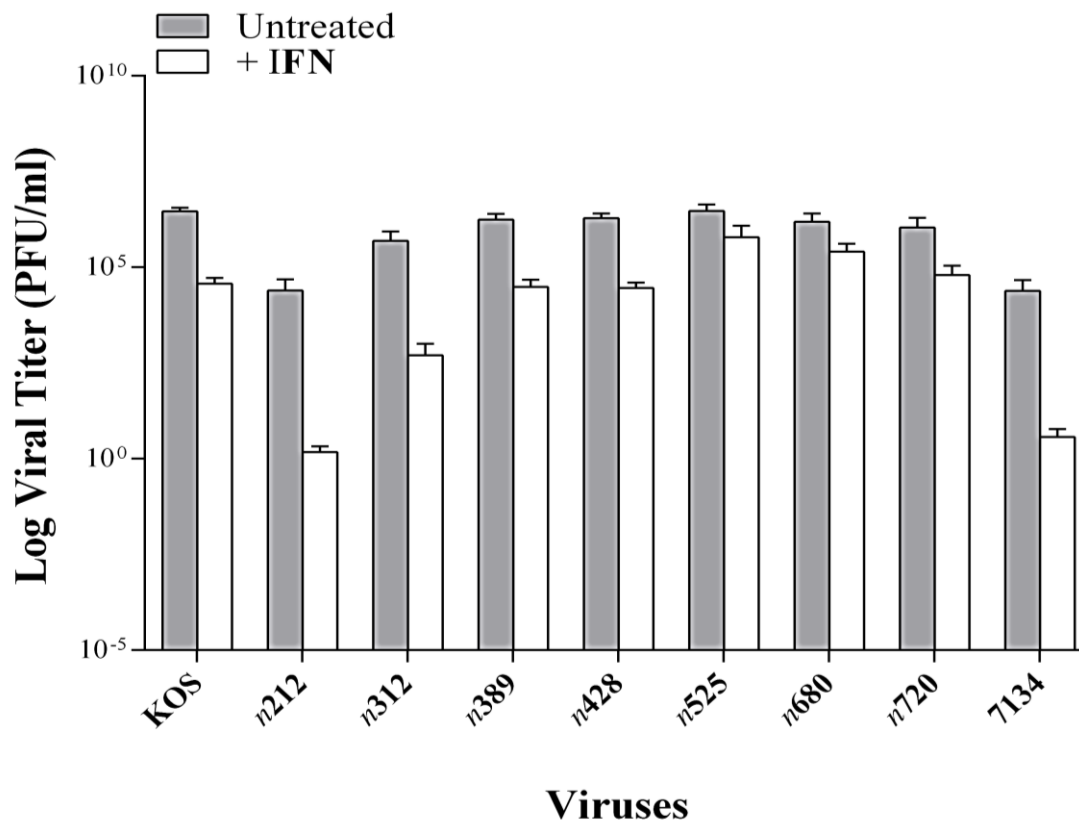


Figure 3-5. ISG15 and ISG56 induction upon IFN- β treatment in HEL and HepaRG cells. HEL cells or HepaRG cells were treated with 1000 U/ml of human IFN- β for the indicated time. At 0 and 24 h post treatment, total RNA was isolated from cells and reversed transcribed into cDNA for qRT-PCR analysis to monitor ISG15 and ISG56 transcript levels upon IFN- β induction. n= 2 experiments.



Ratio	83	≥9273	≥12809	354	114	123	21	61	≥32513
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Figure 3-6. Viral Yields of KOS, 7134 and ICP0 truncation mutants in HepaRG cells in the presence and absence of IFN- β . HepaRG cells were pretreated or not with IFN- β (1000 U/mL) and were infected 15 post-treatment with KOS, 7134 or ICP0 truncation mutants at 1 PFU/cell. Samples were harvested 24 hpi and titered by plaque assays. $n \geq 4$ experiments. Error bars represent the standard errors of the means.

When we used both cell types in viral growth assays, HEL cells were unfortunately very sensitive to acid washes, which we include in this experiments to inactivate extracellular virus. Given this difficulty, we performed our yield assays in HepaRG cells (Figure 3-6).

Results obtained in these assays correlated well with the plaque reduction assays in Figures 3-2, 3-3, and 3-5, distinguishing the viral replication phenotypes of *n312* and *n389*. For this set of experiments the inhibition of viral growth with IFN- β for KOS and *n720* and showed similar reductions, with *n680* being slightly less sensitive to IFN- β than KOS. Reductions for *n525* and *n428* were only ~1.5-fold lower with compared to KOS (Figure 3-6). Lastly, the plaquing of *n389* was at least reduced 4.3-fold with respect to KOS, whereas decrease in *n312* plaquing was 154-fold. We were unable to detect viral replication for *n212* and 7134 in the presence of IFN- β for approximately half of the experiments we performed. These data indicate the first 388 N-terminal amino acids of ICP0 expressed by HSV-1 are sufficient to allow for appreciable viral replication when the type 1 IFN response has been activated in HepaRG cells.

3.4.3. PML Depletion Marginally Increased the Plaquing Efficiency of ICP0 Truncation Mutants in the Presence of INF- β

As previously mentioned in Chapter 2 [91], we identified a domain in ICP0 known ability to promote PML degradation and dissociation of PML and Sp100 from ND10 structures. Given our initial studies showed that overlapping regions impair the IFN response and affected the dispersal of PML (an ISG), we wanted to examine PML's contribution in the IFN response as it related to our ICP0 structure-function studies. It was previously shown that depletion of one or two specific PML isotypes only modestly complemented the replication phenotype of an ICP0 null mutant [92,115].

We then asked the questions, does depletion of PML alleviate IFN- β -mediated restriction on *n212* compared to other ICP0 truncation mutants? To answer that question we obtained HepaRG cells and HepaRGshPML cells, which are depleted for PML. After confirming by RT PCR that PML transcript levels were reduced in HepaRGshPML cells (Mirna Perusina Lanfranca and David Davido unpublished data), we performed a set of IFN- β plaque reduction assays in HepaRGshPML cells and parental HepaRG cells using Wt HSV-1, an ICP0 null mutant, and the majority of our ICP0 truncation mutants (Figure 3-7). In the absence of IFN pretreatment, there was no apparent complementation in the

HepaRGshPML cells (Fig. 3-8). We then performed plaque reduction assays in the mock- or pre-treated IFN- β cultures.

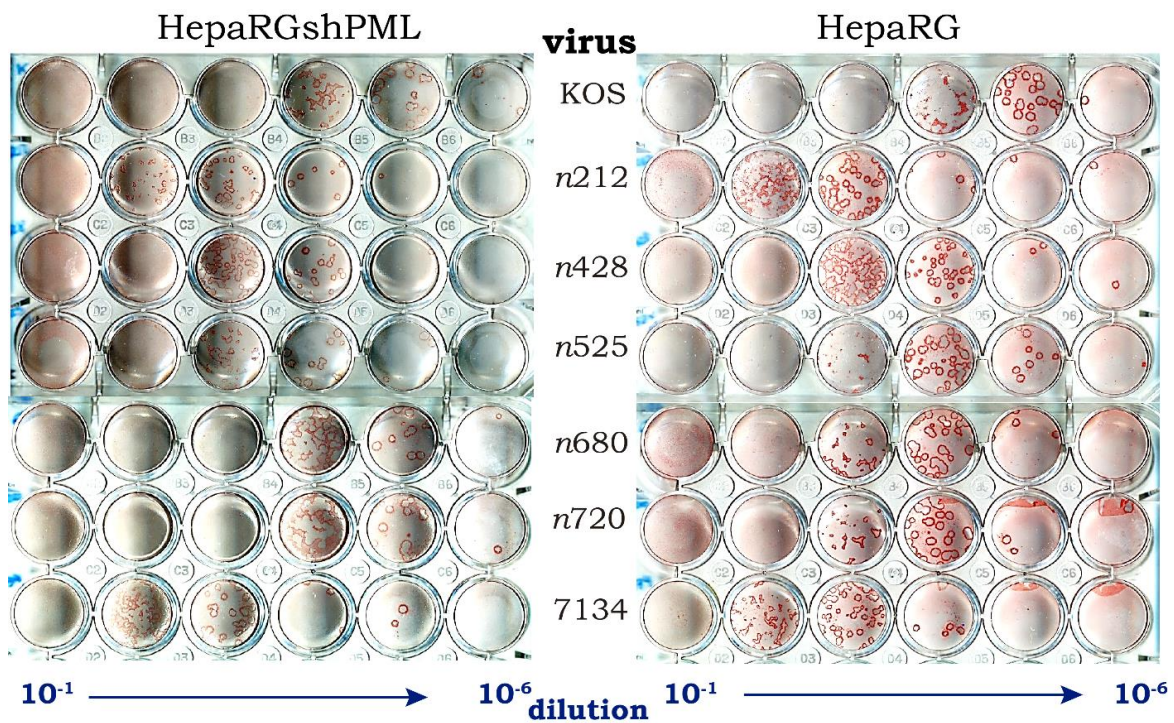


Figure 3-7. Plaquing with KOS, 7134 and ICPO truncation mutants in HepaRGshPML and HepaRG cells. Cells were plated in 24-well plates and infected with 10-fold serial dilutions as indicated, of the respective Wt (KOS), ICPO null (7134) and ICPO truncation mutant viruses. 3 dpi cells were fixed and stained by Immunohistochemistry with a polyclonal antibody against HSV-1. Individual viral plaques are shown in red.

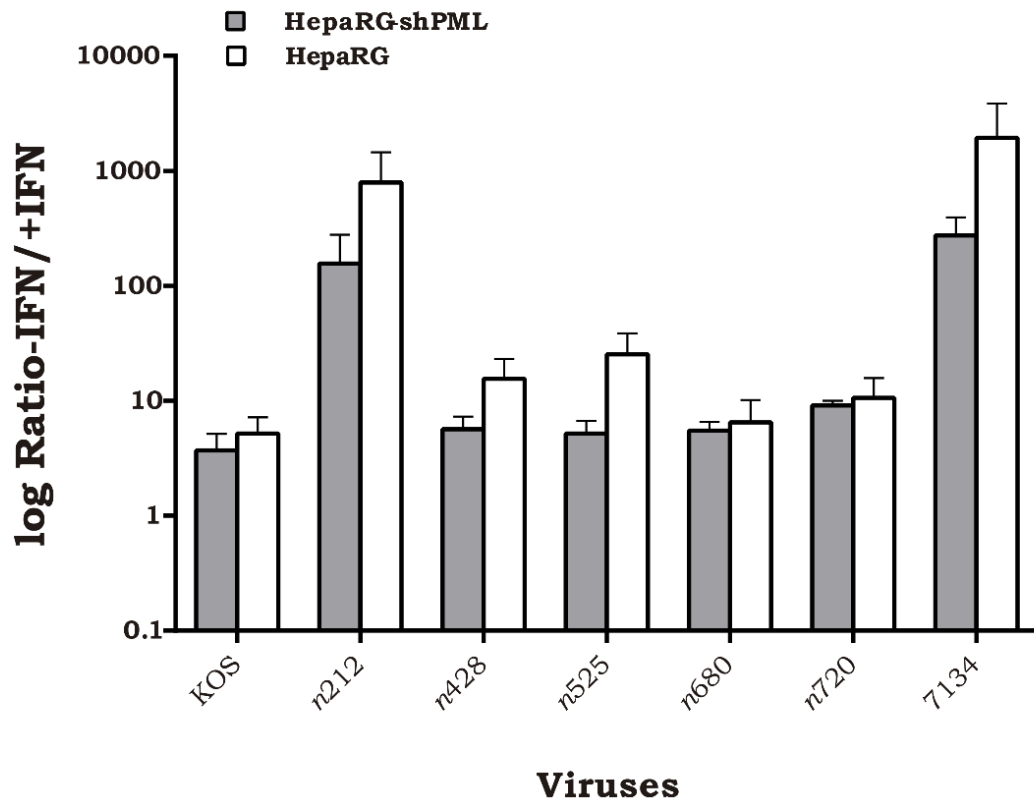


Figure 3-8. Plaque reduction assays of KOS, 7134 and ICP0 truncation mutants in HepaRGshPML vs HepaRG cells in the presence and absence of IFN- β . HepaRGshPML and HepaRG cells were mock or pre-treated or not with IFN- β (1000 U/mL) and infected 15 post-treatment with serial dilutions of KOS, 7134 or ICP0 truncation mutants. 3 dpi cells were fixed and stained by Immunohistochemistry with a polyclonal antibody against HSV-1. Titers were determined as PFU/ml for all viruses. The ratio -IFN/+IFN is the viral titer for a virus in the absence divided by its titer in the presence of IFN- β . n= 3 experiments. Error bars represent the standard errors of the means.

As shown in Figure. 3-8, the lack of PML in HepaRG cells modestly compensated (3-7-fold) for the plating efficiencies of *n212*, *n428*, *n525*, and 7134, as indicated by the decrease in the plating efficiency ratio of these viruses. Thus, our data indicate that PML only contributes in a limited fashion, at best, to the restriction of IFN- β on HSV-1 growth mediated for the ICP0 null and select ICP0 mutant forms we tested. In addition, the results do not precisely correlate with our ICP0 mutant and PML degradation and dissociation data in Chapter 2, suggesting that these two functions of ICP0 (resistance to IFN and PML degradation) are not completely coupled to one another.

3.4. DISCUSSION

HSV-1 establishes a life-long infection in the host, which means that the ability of the virus to evade the immune system is crucial for its survival. Similar to other viruses, HSV-1 expresses viral gene products that counteract host defense mechanisms. The IE protein, ICP0, is a pivotal player in allowing HSV-1 to overcome host antiviral immunity, one part of this immunity being the type I IFN response. As previously mentioned ICP0 null viruses have been shown to be hypersensitive to IFNs [78,79]. ICP0 is able to alter the activation of the IFN regulatory factor 3 (IRF3) [126,141,142], which participates in activating the transcription of IFN- β , although the mechanism of how ICP0 inhibits IRF3 activation is not completely understood.

Many functional regions have been characterized within ICP0, including a RING domain, transactivation domains, NLS, domains related to localization to ND10s (Figure 3-1). Nothing is known about what domains in ICP0 are involved in its ability to thwart the type 1 IFN response for the benefit of HSV-1 replication. To begin to understand how ICP0 impairs the IFN response, we performed structure-function analyses using a series of viruses containing

progressive C-terminal truncations of ICP0 with the goal to identify at least one domain being important for this function.

Monitoring viral fitness by plaque reduction assays in the presence of IFN- β , we observed that there was a graduation in the ability of these mutants to efficiently replicate, which was inversely proportional to the size of ICP0 expressed (Figures 3-2 through 3-5). Specifically, studies show that 7134 and the two smallest N-terminus truncation mutants (*n*212 and *n*312) were unable to produce detectable plaques in HEL cells stimulated with IFN- β and the degree of IFN- β inhibition was not as severe for *n*389-*n*720. The plaquing deficiencies for the initial set of ICP0 mutants we tested were negated on the ICP0 complementing (L7) cells, indicating that the phenotypes we observed were a result of altering ICP0 function. Our viral yield assays essentially mirrored the plaque reduction assay (Fig. 3-6) and we determined that PML only plays a minor in the sensitivity of ICP0 mutants to IFN- β . These data highlight that the region up to amino acids 388 as being critical for HSV-1 to efficiently replicate in the presence of IFN- β , with the residues from 212 to 388 playing a relevant role in this phenotype.

4. CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

HSV-1 is a pathogen that has infected humans for millions of years. Co-evolution between host and virus over the centuries has resulted in a pathogen that is able to establish a persistent infection for the lifetime of a host. As part of this evolution, the host has developed several antiviral immune mechanisms to control viral replication and spread. These mechanisms include intrinsic defenses (see Figure 4-1), such as ND10 and the cellular protein, PML, which are cellular factors that are always present and ready to repress viral transcription or replication [65]. Another arm of the antiviral resistance is the innate immunity (Figure 4-1), where particular molecules or patterns associated with viral replication activate a gene cascade that express effector proteins [66]. This activation in host gene expression leads to the synthesis of type I IFNs, which present a major defense mechanism against viruses' growth [73-75].

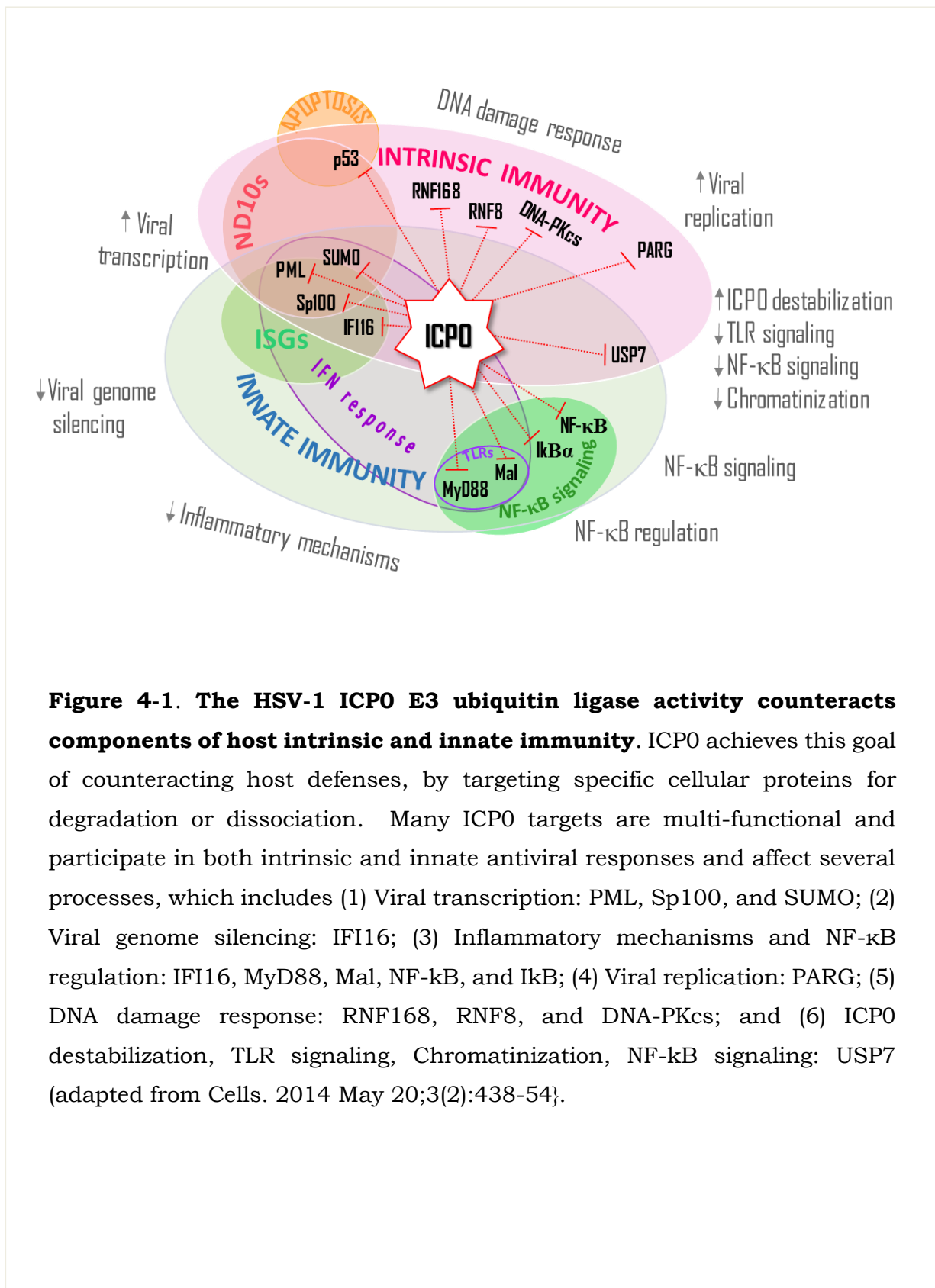


Figure 4-1. The HSV-1 ICP0 E3 ubiquitin ligase activity counteracts components of host intrinsic and innate immunity. ICP0 achieves this goal of counteracting host defenses, by targeting specific cellular proteins for degradation or dissociation. Many ICP0 targets are multi-functional and participate in both intrinsic and innate antiviral responses and affect several processes, which includes (1) Viral transcription: PML, Sp100, and SUMO; (2) Viral genome silencing: IFI16; (3) Inflammatory mechanisms and NF-κB regulation: IFI16, MyD88, Mal, NF-κB, and IκB; (4) Viral replication: PARG; (5) DNA damage response: RNF168, RNF8, and DNA-PKcs; and (6) ICP0 destabilization, TLR signaling, Chromatinization, NF-κB signaling: USP7 (adapted from Cells. 2014 May 20;3(2):438-54).

HSV-1 in turn has acquired counter-defenses to ensure its propagation in the human population. One key component of these defenses is the multifunctional viral protein, ICP0. ICP0 possesses E3 Ub ligase activity, through which directs the proteasomal-dependent degradation (directly or indirectly) of several viral inhibitory function of the host (summarized in Figure 4-1). ICP0 disrupt ND10 structures by directing the degradation of PML, the main organizer of ND10. ICP0 has also been shown to impair a pre-existing type 1 IFN response. The exact molecular mechanisms of how ICP0 inactivates PML and an established type 1 IFN response are still unclear. The theme of this dissertation is to begin to understand how HSV-1 through ICP0 counteracts or manipulates intrinsic (PML/ND10) and innate (IFN) defenses. While select functional domains in ICP0 have been known to play a role in these counter-measures, I hypothesized that other regions in ICP0 contribute to its inactivation of PML/Sp100 and the type 1 IFN, IFN- β . My approach to address this overall hypothesis was to do structure-function analyses using a series of progressively shorter ICP0 C-terminal truncation mutants (Figures 2-1 and 3-1) [91,143].

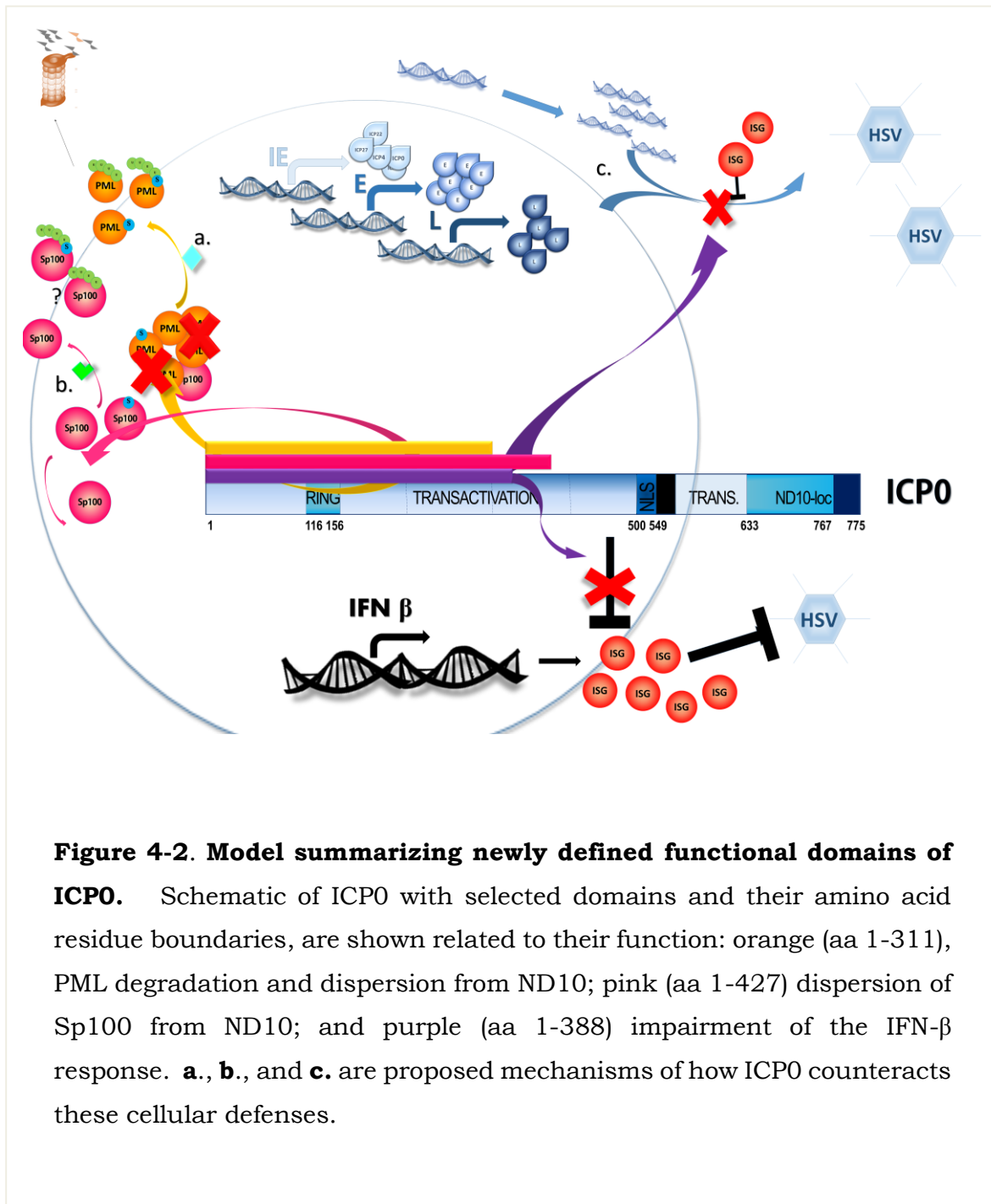
ICP0 potentially exerts these functions within the defined regions of interest, with one or more enzymes or proteins that mediate the degradation or dispersal of ND10 components (Figure 4-2 a. and b.). ICP0 motifs contained in the broad region (residues 212-427) of ICP0 are phosphorylated regions I

and II, and a SIM (Figures 2-1 and 3-1), and each motif has been reported to play a role in ICP0's ability to promote PML and/or Sp100 dissociation from ND10s [44,129,130]. Thus, one plausible hypothesis is that these post-translational modifications or SIM aid in ICP0's ability to bind to one or more cellular adaptor/effectors (Figure 4-2a.). These interactions promotes the degradation and dissociation of SUMOylated-PML and the dissociation of Sp100 from ND10, tagging them for proteasomal degradation. One exception is the major isoform of PML, PML I, which has been shown to interact with ICP0 in co-immunoprecipitation assays and yeast two-hybrid assays [45]. ICP0 can mediate the degradation of a non-SUMOylated form of PML I. These data suggest that ICP0 can directly interact with and ubiquitinate PML I, providing a potential mechanism in which the portions of the N-terminus of ICP0 interacts with PML I in a SUMO-independent manner.

In the case of Sp100 a different domain (residues 1 to 427) within ICP0 seems to be necessary for ICP0's ability to dissociate this ND10 component for the complex versus PML dissociation and degradation, suggesting that ICP0 likely uses additional mechanisms to alter Sp100 localization (Figure 4-2b.).

In Chapter 3, we discovered that the first 388 N-terminal amino acids of ICP0 are required for HSV-1 effectively resist the antiviral effects of IFN- β , with residues spanning from 212-388 important for this function of ICP0 (see

Figure 4-2). As the mutant forms of ICP0, n212 and n312, were expressed at very low levels in the presence of IFN- β (data not shown), it is plausible that the depletion of these proteins adversely impacts viral replication, similar to an ICP0 null mutant. For these two mutants, we propose that these mutant forms of ICP0 may be unable to impair the IFN response because they are not capable of activating transcription from the ICP0 promoter, as well as other HSV-1 promoters. In addition, it has been previously shown that Wt HSV-1 replication in the presence of IFN- β can reduce viral replication, which correlates with a reduction in viral DNA replication [144]. Consequently, we hypothesize that the residues from 1 to 388 of ICP0 promote DNA synthesis or another step late in the viral life cycle by disabling IFN effectors (through their degradation or re-localization), leading to the robust production of infectious viral progeny (Fig. 4-2c.).



Given that I have defined regions in HSV-1 ICP0 that affect two components (PML/ND10 and IFN) of cellular defenses, are similar functions or regions conserved in other ICP0 homologs? It has been noted that varicella-zoster virus (VZV) ICP0 homolog, ORF61 protein, can complement the replication of an HSV-1 ICP0 null mutant [145,146]. Unlike ICP0, ORF61 protein was not able to promote the degradation of PML and only moderately affected Sp100 protein levels [146]. Another comparative study examined the functions of ICP0 homologs from BHV-1 (bovine herpes virus type 1), EHV-1 (equine herpes virus type 1), PRV (pseudorabies virus), and VZV (varicella-zoster virus). These homologs possessed transactivation and *in vitro* ubiquitination activities of ICP0 and could complement and reactivate from quiescent infection an ICP0 null mutant [147] for the first three viruses. However, these ICP0 orthologs differentially affected ND10-associated proteins and did not appear to interfere with type 1 IFN signaling pathways [147]. Lastly, outside of the RING finger domain, there is low level amino acid sequence homology between these viral orthologs and ICP0 [123]. Collectively, these data strongly suggest the regions we identified in HSV-1 ICP0 that modulate host defenses are not conserved among ICP0 homologs, indicating that these other herpesviruses have likely evolve diverse mechanisms to counteract similar host defenses.

As we have examined the interactions between ICP0 and PML/ND10 and ICP0 and IFN- β in this dissertation, the role(s) these cellular factors play in the establishment of, or reactivation from, latency have not been extensively studied. PML is known to be expressed in neurons, and it has been shown that can form a structure surrounding viral genomes [148]. During latency HSV-1 genomes remain chromatinized in the neuronal nucleus as episomes, and no infectious virus is detected. Recent work has suggested that ND10s are responsible, together with centromeres, of associating with HSV-1 genomes, which alters their location and correlates with its silenced transcriptional status, and negatively regulates expression of the LATs [148]. ICP0's impaired ability to disrupt ND10s and centromeres in one differentiated neuronal cell line correlated well with diminished levels of gene expression and replication [149]. From these studies, we can hypothesize that ICP0's ability to disrupt ND10s enhances HSV-1 reactivation in neurons, assuming ND10s repress lytic gene expression.

Previous studies have implicated type 1 signaling as a relevant defense mechanism in neuronal cells from the trigeminal ganglia [150]. ICP0 mutants showed to be severely impaired to replicate and establish latency or reactivate in animal models, implying a role for ICP0 in these functions [151]. Additionally, it was shown that suppression of ICP0 expression correlated with entry into a quiescent state, despite the fact that other IE proteins were still

being made within the cell, supporting a link between ICP0 and quiescence [152]. Since ICP0 null mutants are hypersensitive to IFNs [78], the relationship HSV-1 and IFNs has been investigated to determine the role of the IFN response in latency. It was noted that certain IFN studies were performed in cells that were non-fully differentiated, whereas a new report examined the effects of IFN- β in neurons derived from adult mice [153]. Data from this publication suggest that activation of type 1 IFN signaling alone is not sufficient to restrict Wt HSV-1 replication in adult neurons, and that this is counteracted at least by the viral factor ICP34.5. A study by Halford and co-workers determined that the ICP0 truncation mutant, *n212*, was able to infect severely immunodeficient mice and establish an efficient latent infection [154], suggesting that type 1 IFN signaling plays an important role in the establishment of latency. Consequently, the role of type 1 IFNs in HSV-1 latency and/or reactivation remains to be resolved. The characterization of ICP0 mutants used in our study as it relates to IFN- β signaling in cell culture and *in vivo* will likely shed light on whether ICP0 inactivates the type I IFNs in neurons or vice versa or whether ICP0 and type I IFNs have independent functions from one another to establish a latent infection or reactivate from latency.

In Figure 4-1 several known targets of ICP0's E3 ubiquitin ligase activity are summarized, giving one idea of the breadth of how ICP0 counteracts host

defenses. Known targets of ICP0 ubiquitin-proteasomal degradation, directly or indirectly, include: PML [39-46]; Sp100 [39,40,46,47], CENP-A, -B, -C [48-51], DNA-PKcs [47,52,53], RNF-8 and RNF-168 [53], CD83 [54], USP7[55,56], I κ B α [57], p53 [58], IFI16 [59,60] and E2FBP1 [61], in addition to certain SUMOylated targets [62]. However, ubiquitination of cellular repressors of HSV-1 replication by ICP0 may not be exclusively linked to proteolysis. It is well established that E3 Ub ligases can facilitate the ubiquitination of cellular targets by conjugation of mono-, multi-, and poly-Ub chains. These conjugations can affect a target protein's function, localization, and/or stability [37]. To the best of our knowledge, the possibility that ICP0's exploits other forms of ubiquitination to control the function of cellular factors is just beginning to be explored [64]. Thus, it is logical that a protein as pivotal as ICP0 in the HSV-1 life cycle would utilize non-proteosomal ubiquitination of cellular factors to inactivate their antiviral functions.

In conclusion, ICP0 is a fascinating multi-functional protein. This IE viral protein will provide new insights of antiviral defenses as we begin to understand details on the mechanisms of how HSV-1 utilizes ICP0 to evade the host immunity.

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